PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
George Barrie Kitto and
Mary Susan Burnett

Serial No.: 09/244, 195

Filed: February 4, 1999

For: LIVE VACCINE FOR HUMAN IMMUNODEFICIENCY VIRUS

Group Art Unit: 1674

Examiner: Jeffrey S. Parkin

Atty. Dkt. No.: D6073/CLFR:167US

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David L. Parker

BRIEF ON APPEAL

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BRIEF ON APPEAL

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Sir:

Appellants hereby submit an original and two copies of this Appeal Brief in response to the Final Office Action dated January 26, 2005. The fee for filing this Appeal Brief is attached hereto. This Brief is filed pursuant to the Notice of Appeal mailed July 19, 2005. The date for filing the instant Brief is September 21, 2005, based on the receipt of the Notice of Appeal by the Patent and Trademark Office on July 21, 2005. A petition for a one-month extension under §1.136(a) is included herewith. No additional fees are believed due in connection with the instant paper. However, should any other fees be due, or the attached fee be deficient or absent, the Commissioner is authorized to withdraw the appropriate fee from Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/CLFR:167US/DP01982. Please date stamp and return the enclosed postcard to evidence receipt of this document.

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I. REAL PARTIES IN INTEREST

The real party in interest is Research Development Foundation.

II. RELATED APPEALS AND INTERFERENCES

Appealed U.S. Application 09/632,305, which is related to the above-mentioned application, may affect or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF THE CLAIMS

Claims 1-10 were filed with the original specification. Claims 11-13 were added and claims 1-5, 7, and 11 cancelled in the instant application. Claims 6, 9, 10, 12, and 13 were pending at the time of the final Office Action and are currently pending in the case. The final rejection of claims 6, 8-10, 12 and 13 is the subject of the instant appeal. A copy of the appealed claims is attached hereto as Appendix 1.

IV. STATUS OF AMENDMENTS

Appellants are filing an amendment to the claims pursuant to 37 C.F.R. § 1.116 concurrently with this brief. Appellant believes that the amendment is entitled to entry under the standard set forth in MPEP §1207.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The independent claim on appeal is directed towards a method of initiating both a humoral and a cell-mediated immune response in an animal to an HIV antigen (Specification p. 38, lines 13-16). More specifically, the HIV antigens specified in the appealed independent claim are reverse transcriptase (Specification p. 11, lines 3-4) or transactivating protein

(Specification p. 11, lines 17-19) that are expressed as a fusion construct that contains a gene for surface exposure (Specification p.10, lines 12-15; Specification p.20, lines 3-12). The fusion construct may be expressed in an attenuated bacterium (Specification p.20, lines 13-16; Specification p. 47, lines 11-14), and the attenuated bacterium containing the fusion construct may be administered to an animal to produce both a humoral and a cell-mediated response to the reverse transcriptase or the transactivating protein (Specification p. 6, lines 5-14).

The attenuated bacteria may be *Salmonella* SL3261. Specification p. 7, line 20. The immune response may comprise both a mucosal IgA response and a T cell response. Specification p. 25, lines 19-21. Specification p. 39, lines 9-13. Specification p. 52, lines 1-3. Specification p. 52, lines 16-20. The attenuated bacteria may be administered orally at a dose of about 10^{10} to 10^{14} CFU. Specification p. 26, lines 2-3. OmpA may be used for surface exposure on the attenuated bacterium of the reverse transcriptase or the transactivating protein. Specification p. 20, lines 6-9.

VI. ISSUE ON APPEAL

A. Are claims 6, 8-10, 12 and 13 properly rejected under §112, second paragraph?

B. Are claims 6, 8-10, 12 and 13 obvious under 35 USC § 103(a) over Brey *et al.* in view of each of Georgiou *et al.* and Thimmig *et al.*?

C. Are claims 6, 8-10, 12 and 13 obvious under 35 USC § 103(a) over Hone *et al.* in view of each of Georgiou *et al.* and Thimmig *et al.*?

VII. ARGUMENT

A. The Rejection of Claims 6, 8-10, 12 and 13 Under 35 USC § 112, Second Paragraph, Should Be Reversed.

The Examiner initially rejects claims 6, 8-10, 12 and 13 under 35 USC § 112, second paragraph. It is alleged that the term "said transactivating protein" lacks proper antecedent basis. In light of the present Amendment to the claims, this rejection should be reversed. The instant claim 6 clearly refers to a transactivating protein, and "said transactivating protein" has proper antecedent basis.

B. Claims 6, 8-10, 12 and 13 Were Improperly Rejected Under 35 USC § 103(a) Over Brey *et al.* in View of Each of Georgiou *et al.* and Thimmig *et al.*

The Examiner has finally rejected appealed claims 6, 8-10, 12 and 13 under 35 USC § 103(a) over over Brey *et al.* in view of each of Georgiou *et al.* and Thimmig *et al.* It is alleged that Brey *et al.* teaches *S. typhimurium* for the expression of heterologous antigens. The Examiner alleges that Georgiou *et al.* teaches expression of heterologous antigens as fusion constructs on enteric organisms. The Examiner alleges that Thimmig *et al.* teaches the nucleotide sequence of HIV-1 reverse transcriptase. The Examiner alleges that "it would have been *prima facie* obvious to one having ordinary skill in the art to express the HIV-1 reverse transcriptase gene provided by Thimmig *et al.* as a Lpp-OmpA-tat fusion protein of Georgiou *et al.* in the *S. typhimurium* expression system of Brey *et al*".

As evidenced by the below arguments, it is submitted that the Examiner has not put forth "substantial evidence" on the record to support a *prima facie* case of obviousness.

1. Appellants' Remarks - Claims 6, 9, 12 and 13

In order to maintain such a rejection and shift the burden to the Appellant, the Examiner must come forth with relevant and material scientific evidence that supports the allegations that is sufficient to make out a *prima facie* case. The Examiner has not done that here, for the reasons which follow.

a) Substantial Evidence is Required to Uphold the Examiner's Position

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

Finally, "the considerations such as commercial success, long felt but unsolved needs, and the failure of others to invent are relevant to the obviousness inquiry." *Ryko Mfg. V. Nu-Star, Inc.*, 950 F.2d 714, 719 (Fed. Cir. 1991).

b) The Standard for Obviousness

In order to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the

knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference (or references when combined) must teach or suggest all the claim limitations. *Manual of Patent Examining Procedure* § 2142. Moreover, the teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on Appliant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991). When "the motivation to combine the teachings of the references is not immediately apparent, it is the duty of the examiner to explain why the combination of the teachings is proper." MPEP § 2142.

Also, a prior art reference that 'teaches away' from the claimed invention is a significant factor to be considered in determining obviousness. MPEP § 2145, *citing In re Gurley*, 27 F.3d 551, 554 (Fed. Cir. 1994). In fact, a "reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the applicant." *Tec Air Inc. v. Denso Mfg. Michigan Inc.*, 192 F.3d 1353, 1360 (Fed. Cir. 1999) (citing with approval, *In re Gurley*, 27 F.3d 551 (Fed. Cir. 1994).

c) The Examiner Has Not Demonstrated Any Suggestion or Motivation to Combine the References

Brey *et al.* (Exhibit B) is directed exclusively towards the treatment of malaria, not viruses. Brey *et al.* does not contemplate or disclose any suggestion or motivation to produce a treatment for a virus. Nor does Brey *et al.* contemplate or disclose any suggestion or motivation to produce a vaccine for a virus. Brey *et al.* does not contemplate the treatment of HIV-1. Brey *et al.* does not disclose a transformed attenuated bacterium with a fusion protein comprising a

viral antigen. Further, Brey *et al.* does not contemplate the generation of a HIV-1 vaccine by expressing HIV-1 reverse transcriptase or transactivating protein as a fusion protein on an attenuated bacterial host. There is further no indication in Brey *et al.* that expression of a viral antigen would necessarily result in both a cellular and humoral immune response. Brey et al. does not teach all of the claim limitations, as required by MPEP§2143.

Appellants have evaluated Georgiou *et al.* (Exhibit C), and the only mention of HIV-1 that Appellants have identified in Georgiou *et al.* is a single sentence describing the prior use of the HIV-1 gp 120 glycoprotein (col. 13, lines 29-32). The very next sentence of Georgiou *et al.* states "However, the presence of a fragment of a protein from an infectious agent does not give satisfactory protection against disease" (col 13, lines 32-35). Georgiou *et al.* does not disclose the use of transactivating protein or reverse transcriptase.

Georgiou *et al.* does not suggest to express an HIV-1 protein on a attenuated bacterial host to induce both a humoral and a cellular immune response. Georgiou *et al.* does not disclose the HIV-1 proteins reverse transcriptase or transactivating protein. Appellants agree with the Examiner's statement that Georgiou *et al.* does not disclose recombinants expressing the HIV-1 reverse transcriptase gene (Exhibit 1, p.3). Additionally, unlike the present Application (*e.g.*, p. 14, line 6 to p.15 line 2), there is no suggestion in Georgiou *et al.* that the expression system presented therein might be used for generating an immune response against HIV-1. In contrast, the present invention discloses that a transformed attenuated bacteria comprising an HIV-1 protein is particularly attractive for generating an immune response towards HIV-1 (see, *e.g.*, p. 14 line 6 - p. 15 line 2).

Thus, There is no suggestion in Georgiou *et al.* to modify the protocol in order to express an HIV-1 protein on a attenuated bacterial host to induce both a humoral and a cellular immune response, as required by MPEP §2143. Further, Georgiou *et al.* does not teach all of the claim limitations, as required by MPEP §2143.

Thimmig *et al.* (Exhibit D) is concerned with the identification of the nucleotide sequence of reverse transcriptase, not the generation of an HIV-1 vaccine. There is no indication in Thimmig *et al.* that HIV-1 reverse transcriptase could be used in a vaccine. There is no suggestion or indication in Thimmig *et al.* that reverse transcriptase could be expressed in an attenuated bacterium as a fusion construct. Further, Thimmig *et al.* does not contemplate that an attenuated bacterium expressing reverse transcriptase as a fusion construct could be used to generate both a humoral and a cellular immune response. Thimmig *et al.* does not teach all of the claim limitations.

Brey *et al.* is directed exclusively towards the treatment of malaria. Brey *et al.* does not teach all of the claim limitations, and there is no suggestion or motivation to combine Brey *et al.* with Georgiou *et al.* or Thimmig *et al.*

The Examiner has not demonstrated that Brey *et al.*, Georgiou *et al. and* Thimmig *et al.* are properly combinable. Brey *et al.* is directed towards the treatment of malaria (not viruses) and is not combinable with Thimmig *et al.* or Georgiou *et al.* Thimmig *et al.* is directed towards the nucleotide sequence of reverse transcriptase (not the creation of a vaccine or the generation of an immune response); Thimmig *et al.* does not does not suggest the desirability of combination with either Georgiou *et al. or* Brey *et al.* Georgiou *et al.* may provide a general

approach for expressing a protein for the purpose of inducing an immune response; however Georgiou *et al.* does not contemplate the generation of an HIV-1 vaccine.

d) There is Not a Reasonable Expectation of Success

Georgiou *et al.* additionally discloses the unpredictability associated with attempts to elicit an immune response to an antigen. Georgiou *et al.* states, "However, the presence of a fragment of a protein from an infectious agent often does not give satisfactory protection against disease" (col. 13, lines 32-35). Georgiou *et al.* further states, "Immunization with an intact protein is more likely to elicit a humoral immune response and provide protective immunity" (col.13, lines 38-40). Implicit with this statement is the fact that immunization with an intact protein may *not* result in a humoral immune response. Further, immunization with an intact protein may *not* result in *both* a humoral immune response *and* a cellular immune response. The reference to failed attempts to elicit an immune response which could be helpful for the treatment or prevention of a disease additionally indicates that there would be no reasonable expectation of success, as required by MPEP §2143, to produce both a humoral and cellular immune response to an HIV protein expressed in an attenuated bacterial host.

There is no indication in either Thimmig *et al.* or Brey *et al.* that there would be any reasonable expectation of success, as required by MPEP§2143, to generate both a humoral and a cellular immune response by expressing HIV-1 transactivating protein or reverse transcriptase as a fusion protein on an attenuated bacterium.

e) The References Do Not Teach All of the Claim Limitations

The instant claims refer to the generation of both a humoral and a cellular immune response in an animal to an HIV-1 reverse transcriptase or transactivating protein. In neither Georgiou *et al.*, Thimmig *et al.* or Brey *et al.* is there any mention of the generation of both a

humoral and a cellular immune response to an HIV-1 reverse transcriptase or transactivating protein.

2. Appellants' Remarks - Claim 8

The Examiner has not made a *prima facie* case regarding claim 8 for all of the same reasons regarding claims 6, 9, 12 and 15, as set forth above.

Additionally, there are further reasons that the Examiner has not come forth with relevant and material scientific evidence that supports the allegations that is sufficient to make out a *prima facie* case regarding claim 8. Specifically, claim 8 requires both a mucosal IgA response and a helper T cell response.

The Examiner has not shown any teaching or suggestion of a mucosal immune response in Georgiou *et al.*, Thimmig *et al.* or Brey *et al.* The Examiner has not shown any teaching or suggestion of an IgA immune response in Georgiou *et al.*, Thimmig *et al.* or Brey *et al.* The Examiner has not shown any teaching or suggestion of both a mucosal immune response and a helper T cell response in Georgiou *et al.*, Thimmig *et al.* or Brey *et al.* The Examiner has not shown any teaching or suggestion of both a mucosal IgA immune response and a helper T cell response in Georgiou *et al.*, Thimmig *et al.* or Brey *et al.* Appellants have evaluated Georgiou *et al.*, Thimmig *et al.* and Brey *et al.* and have been unable to identify a single mention of a mucosal immune response or an IgA immune response. Further, Appellants have evaluated Georgiou *et al.*, Thimmig *et al.* and Brey *et al.* and have been unable to identify any mention of both a mucosal IgA immune response and a helper T cell response.

Thus, Georgiou *et al.*, Thimmig *et al.* and Brey *et al.* do not contain all of the claim limitations regarding claim 8.

For these additional reasons, Appellants respectfully request that the rejection of claim 8 be removed.

3. Appellants' Remarks - Claim 10

The Examiner has not made a *prima facie* case regarding claim 10 for all of the same reasons regarding claims 6, 9, 12 and 15, as set forth above.

Additionally, there are further reasons that the Examiner has not come forth with relevant and material scientific evidence that supports the allegations that is sufficient to make out a *prima facie* case regarding claim 10. Specifically, claim 10 requires administration of an attenuated bacterial host in the amount of 10^{12}-10^{14} CFU.

The Examiner has not shown any teaching or suggestion of the administration of an attenuated bacterial host in the amount of 10^{12}-10^{14} CFU in Georgiou *et al.*, Thimmig *et al.* or Brey *et al.* Further, Appellants have evaluated Georgiou *et al.*, Thimmig *et al.* and Brey *et al.* and have been unable to identify any mention of the administration of an attenuated bacterial host in the amount of 10^{12}-10^{14} CFU.

Thus, Georgiou *et al.*, Thimmig *et al.* and Brey *et al.* do not contain all of the claim limitations regarding claim 10.

For these additional reasons, Appellants respectfully request that the rejection of claim 10 be removed.

C. Claims 6, 8-10, 12 and 13 Were Improperly Rejected Under 35 USC § 103(a) Over Hone *et al.* in View of Each of Georgiou *et al.* and Thimmig *et al.*

The Examiner has finally rejected appealed claims 6, 8-10, 12 and 13 under 35 USC § 103(a) over Hone *et al.* in view of each of Georgiou et al. and Thimmig *et al.* It was stated that

Hone *et al.* teaches expression of the gp120 antigen as a fusion construct in attenuated *Salmonella typhimurium*. As stated above, the Examiner alleges that Georgiou *et al.* teaches expression of heterologous antigens as fusion constructs on enteric organisms. It is stated that Thimmig *et al.* teaches the nucleotide sequence of HIV-1 reverse transcriptase. The Examiner alleges that it would have been prima facie obvious to one of skill in the art to express the reverse transcriptase of Thimmig *et al.*, as a Lpp-OmpA-Tat fusion protein of Georgiou *et al.*, in the *S. typhimurium* expression system of Hone *et al.*

4. Appellants' Remarks - Claims 6, 8, 9 and 12

In order to maintain such a rejection and shift the burden to the Appellant, the Examiner must come forth with relevant and material scientific evidence that supports the allegations that is sufficient to make out a *prima facie* case. The Examiner has not done that here, for the reasons which follow.

a) The References Are Not Properly Combinable

Hone *et al.* (Exhibit E) does not suggest or disclose the generation of an immune response to reverse transcriptase or transactivating protein. There is no mention of reverse transcriptase or transactivating protein whatsoever in Hone *et al.* Appellants agree with the Examiner's statement that Hone *et al.* "does not disclose Lpp-OmpA-HIV-1 Tat fusion proteins" (Exhibit A, p. 6). Hone *et al.* does not disclose all of the elements of the invention.

Further, Hone *et al.* does not disclose that both a humoral and a cell-mediated immune response can be generated for any antigen. Hone *et al.* merely *hypothesizes* that a T-cell mediated response *might* be observed for *gp120*. Hone *et al.* states on p.206, "It is reasonable to ***propose***, therefore, that Salmonella bearing ***rgp120*** will elicit gp120-specific $CD8^+$ CTLs" (emphasis added). Hone *et al.* observes ***only*** a ***humoral*** immune response to gp120 (*e.g.*, Table

1). As stated in Hone *et al.* on p.206, "***Presently***, there is ***no consensus*** on the vector configuration that optimizes the ability of Salmonella to induce foreign antigen-specific cytotoxic $CD8^+$ CTLs in vivo" (emphasis added). Further, Hone *et al.* points out failures to generate a cell-mediated immune response to an antigen expressed on a Salmonella, "Antigen-specific $CD8^+$ CTLs were not detected after immunization with recombinant Salmonella vaccine vectors that expressed cytoplasmic influenza A nucleoprotein" (p.206). Hone *et al.* concludes that, "Collectively, these observations suggest that ***individual foreign antigen characteristics*** will dictate the Salmonella vector configuration that optimizes the level of such [cell-mediated] responses" (p.206). Hone does not disclose reverse transcriptase or transactivating protein. Hone *et al.* does not disclose that expression of reverse transcriptase or transactivating protein could be used to generate both a humoral and a cellular immune response.

As stated above, Thimmig *et al.* does not disclose the generation of an immune response to reverse transcriptase. Thimmig *et al.* does not disclose or suggest the expression of reverse transcriptase in an attenuated bacterium. Thimmig *et al.* does not contemplate the creation of an HIV-1 vaccine. Thimmig *et al.* does not disclose or suggest the expression of reverse transcriptase in an attenuated bacterium to produce both a cellular and a humoral immune response. As stated above, Thimmig *et al.* does not disclose all of the claim limitations.

As stated above, Georgiou *et al.* does not disclose or suggest the generation of an immune response to HIV-1 reverse transcriptase or transactivating protein. Thus, the references have not been shown to be combinable and no *prima facie* rejection made on this record. "The mere fact that references can be combined or modified does not render the resultant combination obvious ***unless the prior art also suggests the desirability of the combination***." MPEP § 2143.01 (emphasis added).

b) There is No Reasonable Expectation of Success

Hone et al. demonstrates that there is a large degree of uncertainty in the art--even when evaluating only a single antigen (gp120) which is distinct from the antigens of the instant claims (*i.e.*, reverse transcriptase and transactivating protein). Hone *et al.* clearly states that there is no consensus regarding the degree to which both a cellular and a humoral immune may be generated to an antigen expressed in an attenuated bacterium. Hone *et al.* states on p.206, "***Presently***, there is ***no consensus*** on the vector configuration that optimizes the ability of Salmonella to induce foreign antigen-specific cytotoxic $CD8^+$ CTLs in vivo" (emphasis added).

Hone *et al.* discusses the failure of others to produce a cell-mediated immune response to an antigen expressed in an attenuated bacteria. "Antigen-specific $CD8^+$ CTLs were not detected after immunization with recombinant Salmonella vaccine vectors that expressed cytoplasmic influenza A nucleoprotein" (p.206). Hone *et al.* concludes that, "Collectively, these observations suggest that ***individual foreign antigen characteristics*** will dictate the Salmonella vector configuration that optimizes the level of such [cell-mediated] responses" (p.206).

Hone *et al.* states that there is a long-standing need for an HIV vaccine that can produce both cellular and humoral immune responses. Hone *et al.* states on p.204, "The ***long term objective*** of our group, therefore , is to optimize the capacity of our live oral Salmonella-HIV vaccine vectors to elicit HIV-specific... T cell immunity" (emphasis added).

As stated above, Hone *et al.* only ***hypothesizes*** that a cell-mediated immune response will be observed for gp120 expressed in *Salmonella*. Hone *et al.* states on p.206, "It is reasonable to ***propose***, therefore, that Salmonella bearing ***rgp120*** will elicit gp120-specific $CD8^+$ CTLs" (emphasis added). Implicit with this hypothesis is the possibility that gp120 expressed in

Salmonella might ***not*** produce a cell-mediated immune response. Thus, if uncertainty existed as to whether or not gp120 (a protein for which significant immunological data regarding humoral responses is presented in Hone *et al.*) could be used to generate a cell-mediated immune response, then there would ***necessarily be an even higher degree of uncertainty*** as to whether or not another ***untested*** antigen could be expressed in an attenuated bacterium to produce both a humoral and a cellular immune response. Hone *et al.* shows that there is no reasonable expectation of success.

Additionally, as stated above, Georgiou *et al.* additionally discloses the unpredictability associated with attempts to elicit an immune response to an antigen. Georgiou *et al.* states, "Immunization with an intact protein is more likely to elicit a humoral immune response and provide protective immunity" (col.13, lines 38-40). Implicit with this statement is the fact that immunization with an intact protein may *not* result in a humoral immune response. Further, immunization with an intact protein may *not* result in *both* a humoral immune response *and* a cellular immune response. The reference to failed attempts to elicit an immune response which could be helpful for the treatment or prevention of a disease additionally indicates that there would be no reasonable expectation of success, as required by MPEP §2143, to produce both a humoral and cellular immune response to an HIV protein expressed in an attenuated bacterial host.

Thimmig *et al.* is directed neither towards the generation of an immune response nor to the creation of an HIV vaccine, and Thimmig *et al.* does not provide any reasonable expectation of success, as required by MPEP §2143, to produce both a humoral and cellular immune response to an HIV protein expressed in an attenuated bacterial host.

c) The References Do Not Teach All of the Claim Limitations

The instant claims refer to the generation of both a humoral and a cellular immune response in an animal to an HIV-1 reverse transcriptase or transactivating protein. In neither Georgiou *et al.*, Thimmig *et al.* or Hone *et al.* is there any mention of the generation of both a humoral and a cellular immune response to an HIV-1 reverse transcriptase or transactivating protein.

5. Appellants' Remarks - Claim 10

The Examiner has not made a *prima facie* case regarding claim 10 for all of the same reasons regarding claims 6, 8, 9 and 12, as set forth above.

Additionally, there are further reasons that the Examiner has not come forth with relevant and material scientific evidence that supports the allegations that is sufficient to make out a *prima facie* case regarding claim 10. Specifically, claim 10 requires administration of an attenuated bacterial host in the amount of 10^{12}-10^{14} CFU.

The Examiner has not shown any teaching or suggestion of the administration of an attenuated bacterial host in the amount of 10^{12}-10^{14} CFU in Georgiou *et al.*, Thimmig *et al.* or Hone *et al.* Further, Appellants have evaluated Georgiou *et al.*, Thimmig *et al.* and Hone *et al.* and have been unable to identify any mention of the administration of an attenuated bacterial host in the amount of 10^{12}-10^{14} CFU.

Thus, Georgiou *et al.*, Thimmig *et al.* and Hone *et al.* do not contain all of the claim limitations regarding claim 10.

For these additional reasons, Appellants respectfully request that the rejection of claim 10 be removed.

6. Appellants' Remarks - Claim 13

The Examiner has not made a *prima facie* case regarding claim 13 for all of the same reasons regarding claims 6, 8, 9 and 12, as set forth above.

Additionally, there are further reasons that the Examiner has not come forth with relevant and material scientific evidence that supports the allegations that is sufficient to make out a *prima facie* case regarding claim 13. Specifically, claim 13 requires that the bacterial host is *Salmonella typhiruim* SL3261.

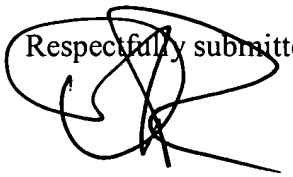
The Examiner has not shown any teaching or suggestion of *Salmonella typhiruim* SL3261 in Georgiou *et al.*, Thimmig *et al.* or Hone *et al.* Further, Appellants have evaluated Georgiou *et al.*, Thimmig *et al.* and Hone *et al.* and have been unable to identify any mention of *Salmonella typhiruim* SL3261.

Thus, Georgiou *et al.*, Thimmig *et al.* and Hone *et al.* do not contain all of the claim limitations regarding claim 13.

For these additional reasons, Appellants respectfully request that the rejection of claim 10 be removed.

VIII. CONCLUSION

It is respectfully submitted, in light of the above, none of the pending claims are properly rejected under 35 U.S.C. §103. Therefore, Appellants request that the Board reverse the pending grounds for rejection.



Respectfully submitted,

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Date: October 21, 2005

APPENDIX 1: APPEALED CLAIMS

6. A method of initiating an immune response specific for transactivating protein or reverse transcriptase of human immunodeficiency virus type 1 (HIV-1) in an animal, said method comprises the step of:

administering to said animal an attenuated bacterial host comprising a recombinant plasmid that carries a fusion protein construct, wherein said fusion protein construct comprises a gene required for surface exposure and a gene encoding said transactivating protein or reverse transcriptase of HIV-1, wherein said bacterial host can induce both cellular and humoral anti-HIV-1 immune responses in said animal.

8. The method of claim 6, wherein said immune response comprises a mucosal IgA response and a helper T cell response.

9. The method of claim 6, wherein said attenuated bacterial host is administered orally.

10. The method of claim 6, wherein said attenuated bacterial host is administered in an oral dose of from about 10^{12} to about 10^{14} CFU.

12. The method of claim 6, wherein said gene required for surface exposure encodes *E. coli* lipoprotein signal sequende linked to a portion of the *E. coli* outer membrane protein ompA.

13. The method of claim 6, wherein said attenuated bacterial host is a strain of *Salmonella typhimurium*, SL3261.

APPENDIX 2: EVIDENCE APPENDIX

Exhibit A: Final Office Action dated January 26, 2005

Exhibit B: Brey *et al.* (U.S. Patent 5,112,749); cited in Office Action dated January 26, 2005.

Exhibit C: Georgiou *et al.* (U.S. Patent 5,348,867); cited in Office Action dated January 26, 2005.

Exhibit D: Thimmig *et al.* (The Journal of Biological Chemistry (1993) vol: 268(22), p. 16528-16536); cited in Office Action dated January 26, 2005.

Exhibit E: Hone *et al.* (Journal of Biotechnology (1996) vol: 44, p.203-207); cited in Office Action dated January 26, 2005.

EXHIBIT A

D6073-11-14

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/244,195	02/04/1999	GEORGE BARRIE KITTO	D6073	3475

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EXAMINER	
PARKIN, JEFFREY S	

ART UNIT	PAPER NUMBER
1648	

DATE MAILED: 01/26/2005

2 mo. due to Revoke Advis

Please find below and/or attached an Office communication concerning this application or proceeding.

Action 3/26/05. Initial
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Date(s) Docketed: Deadline for Final 3
mo. Off due 4/26/05. Final
7/26/05. Notice of Appeal due
4/26/05. Final for Notice of Appeal
FEB 0 1 2005 7/26/05.
Client: CLFR: 167-ALT
Attorney(s): DLP
Initials: JE AW

Office Action Summary	Application No.	Applicant(s)	
	09/244,195	KITTO ET AL.	
	Examiner	Art Unit	
	Jeffrey S. Parkin, Ph.D.	1648	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *03* MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *01 November 2004*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *6,8-10,12 and 13* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *6,8-10,12 and 13* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

Detailed Office Action

Status of the Claims

Acknowledgement is hereby made of receipt and entry of the submission filed 01 November, 2004, wherein claim 6 was amended.[1] Claims 6, 8-10, 12, and 13 are pending in the instant application.

35 U.S.C. § 112, Second Paragraph

Claims 6, 8-10, 12, and 13 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Two separate requirements are set forth under this statute: (1) the claims must set forth the subject matter that applicants regard as their invention; and (2) the claims must particularly point out and distinctly define the metes and bounds of the subject matter that will be protected by the patent grant. Claim 6 recites the limitation "said transactivating protein". There is insufficient antecedent basis for this limitation in the claim.

35 U.S.C. § 103(a)

The following is a quotation of 35 U.S.C. § 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

---

[1] Applicants are advised that the amendment filed 01 November, 2004, failed to include the status of claim 8 as required pursuant to 37 C.F.R. 1.121(c). However, since the claim was included in the arguments section of applicants' response, the examiner will assume that it is still pending.

> Patentability shall not be negatived by the manner in which the invention was made.
>
> Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. § 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 C.F.R. § 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. § 103(c) and potential 35 U.S.C. § 102(f) or (g) prior art under 35 U.S.C. § 103(a).

Claims 6, 8-10, 12, and 13 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Brey *et al.* (1992), in view of Georgiou *et al.* (1994) and Thimmig *et al.* (1993). As previously set forth, Brey *et al.* (1992) describe the preparation of S. *typhimurium* expression systems (including those derived from strain SL3261) that are useful for the expression of heterologous (e.g., malaria) antigens. A detailed description of suitable expression vectors can be found in Table 1 and column 20. This publications also discloses that said expression systems are particularly useful because the vectors of interest retain their enteroinvasive properties but are markedly reduced in terms of virulence. This properties make these vectors particularly useful for generating both humoral and cell-mediated immune responses against the antigen

of interest (see col. 7, first paragraph). Various vaccine formulations can be prepared and routes of administration utilized (i.e., oral, intradermal, intramuscular, intraperitoneal, intranasal, etc.) (see col. 21, section 5.6). A particularly attractive feature of this vector system is the ability of S. *typhimurium* to invade the gut epithelial tissue thereby leading to strong mucosal and helper immune responses (see cols. 23 and 24, section 5.6.2). Other advantages of this vector system include the lack of a necessary purification step for the immunogen of interest and the ability of this system to be inexpensively produced and conveniently administered. The probability of adverse reactions in both animals and humans is also low. This teaching does not disclose the utilization of an Lpp-OmpA-RT fusion protein.

Georgiou *et al.* (1994) describe the preparation of recombinant DNAs that are suitable for the expression of a heterologous antigen on the external surface of an enteric microorganism (e.g., E. *coli* or Salmonella). DNA constructs were prepared that were capable of encoding fusion proteins comprising the Lpp signal sequence, OmpA coding portion, and a heterologous antigen (i.e., see cols. 3, 4, 15, and Figure 1). The inventors noted that targeting sequences (e.g., Lpp) and membrane traversing amino acid sequences (e.g., OmpA) are well-known in the prior art (see cols. 3 and 4). The inclusion of these coding sequences in a fusion construct facilitates the expression, transport, and presentation of a heterologous antigen on the cell surface of a gram-negative bacterium. It was reported that various strains of Salmonella would prove particularly useful for the invention (see col. 5, last paragraph). This teaching does not disclose recombinants expressing the HIV-1 *reverse transcriptase* gene.

Thimmig and colleague provide the complete nucleotide/amino acid sequence of the HIV-1 RT gene and expression vectors comprising

said gene. For instance, see MATERIALS AND METHODS, p. 16529, and Results, pages 16530-16533, wherein the gene, expression vectors, and cell lines producing said protein are described. Thus, this teaching clearly illustrates that HIV-1 RT was widely available and of obvious diagnostic and medical importance.

Therefore, it would have been *prima facie* obvious to one having ordinary skill in the art at the time the invention was made to express the HIV-1 RT gene provided by Thimmig *et al.* (1993), as an Lpp-OmpA-Tat fusion protein, as suggested by Georgiou *et al.* (1994), in the S. *typhimurium* expression system described by Brey *et al.* (1992), since Brey and colleagues teach that this system is useful for generating strong immune responses against the antigen of interest. The skilled artisan would have been motivated to prepare such constructs since this would facilitate the development of HIV-1 RT-specific immunological reagents (i.e., antibodies) which can be employed in diagnostic, immunological, or biochemical assays. It would have also been *prima facie* obvious to one having ordinary skill in the art at the time the invention was made to prepare a fusion protein comprising the Lpp signal sequence, OmpA, and HIV-1 RT since Georgiou *et al.* (1994) teach that Lpp-OmpA-X fusion proteins are expressed in large quantities in an antigenic/immunogenic form on the cell surface of enteric bacteria.

Response to Arguments

Applicants traverse and submit that the prior art fails to teach or suggest fusion constructs employing RT. This argument is clearly not persuasive. The examiner recognizes that references cannot be arbitrarily combined and that there must be some reason why one skilled in the art would be motivated to make the proposed combination of primary and secondary references. *In re Nomiya*, 184 U.S.P.Q. 607 (C.C.P.A. 1975). However, there is no requirement that

a motivation to make the modification be expressly articulated. The test for combining references is what the combination of disclosures taken as a whole would suggest to one of ordinary skill in the art. *In re McLaughlin*, 170 U.S.P.Q. 209 (C.C.P.A. 1971). References are evaluated by what they suggest to one versed in the art, rather than by their specific disclosures. *In re Bozek*, 163 U.S.P.Q. 545 (C.C.P.A. 1969). In this case, the prior art clearly provides efficient tools for making large quantities of heterologous proteins that retain the natural conformations and biochemical activities. The only issue is whether one of ordinary skill in the art would have been motivated to prepare RT fusion constructs using these reagents. The answer to this question is emphatically yes. The human immunodeficiency virus type 1 is the aetiological agent of AIDS. One of ordinary skill in the art would have had more than sufficient motivation to prepare RT fusion constructs because of the medical importance of this virus and gene product. Utilizing the Salmonella expression system would provide a facile means for generating large quantities of an immunogenic protein that will be capable of inducing both humoral and cell-mediated immune response.

Claims 6, 8-10, 12, and 13[2] are rejected under 35 U.S.C. § 103(a) as being obvious over Hone *et al.* (1996) in view of Georgiou *et al.*

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[2] As previously set forth, the teachings of Hone and colleagues describes the use of an S. *typhimurium* strain carrying a mutation in the *aro* locus. This attenuated bacterial strain appears to be the same strain described by Fouts *et al.* (1995, Construction and immunogenicity of *Salmonella typhimurium* vaccine vectors that express HIV-1 gp120, Vaccine, 13(17):1697-705) which was designated strain SL3261. Since the Patent Office does not have the facilities for examining and comparing applicants' claimed S. *typhimurium* strain SL3261 with the S. *typhimurium* strain employed by Hone *et al.* (1996), the burden is upon applicants to demonstrate the unobvious genotypic/phenotypic differences between the two strains. *In re Best*, 562 F.2d 1252, 195 U.S.P.Q. 430 (C.C.P.A. 1977). *Ex parte Gray*, 10 U.S.P.Q.2d 1922 (Bd. Pat. Appl. Int. 1989).

(1994) and Thimmig *et al.* (1993). Hone and colleagues provide attenuated *Salmonella typhimurium* vaccine vectors containing expression vectors encoding *Escherichia coli* OmpA::HIV-1 gp120 fusion proteins. These Salmonella strains induced both mucosal and systemic HIV-1 gp120-specific immune responses. The authors concluded (see Abstract, p. 203) that "These results, therefore, support the proposal that *Salmonella* vectors will be a safe and inexpensive means for delivery of HIV antigens to, and the elicitation of HIV-specific T cells in, the mucosal and systemic compartments." The authors also noted (p. 206, penultimate paragraph) that "It is reasonable to propose, therefore, that *Salmonella* bearing surface-expressed rgp120 will elicit gp120-specific $CD8^+$ CTLs." This teaching does not disclose Lpp-OmpA-HIV-1 Tat fusion proteins.

Georgiou *et al.* (1994) describe the preparation of recombinant DNAs that are suitable for the expression of a heterologous antigen on the external surface of an enteric microorganism (e.g., E. *coli* or Salmonella). DNA constructs were prepared that were capable of encoding fusion proteins comprising the Lpp signal sequence, OmpA coding portion, and a heterologous antigen (i.e., see cols. 3, 4, 15, and Figure 1). The inventors noted that targeting sequences (e.g., Lpp) and membrane traversing amino acid sequences (e.g., OmpA) are well-known in the prior art (see cols. 3 and 4). The inclusion of these coding sequences in a fusion construct facilitates the expression, transport, and presentation of a heterologous antigen on the cell surface of a gram-negative bacterium. It was reported that various strains of Salmonella would prove particularly useful for the invention (see col. 5, last paragraph). This teaching does not disclose recombinants expressing the HIV-1 *tat* gene.

Thimmig and colleague provide the complete nucleotide/amino acid

sequence of the HIV-1 RT gene and expression vectors comprising said gene. For instance, see MATERIALS AND METHODS, p. 16529, and Results, pages 16530-16533, wherein the gene, expression vectors, and cell lines producing said protein are described. Thus, this teaching clearly illustrates that HIV-1 RT was widely available and of obvious diagnostic and medical importance.

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U.S.P.Q. 607 (C.C.P.A. 1975). However, there is no requirement that a motivation to make the modification be expressly articulated. The test for combining references is what the combination of disclosures taken as a whole would suggest to one of ordinary skill in the art. *In re McLaughlin*, 170 U.S.P.Q. 209 (C.C.P.A. 1971). References are evaluated by what they suggest to one versed in the art, rather than by their specific disclosures. *In re Bozek*, 163 U.S.P.Q. 545 (C.C.P.A. 1969). In this case, the prior art clearly provides efficient tools for making large quantities of heterologous proteins that retain the natural conformations and biochemical activities. The only issue is whether one of ordinary skill in the art would have been motivated to prepare RT fusion constructs using these reagents. The answer to this question is emphatically yes. The human immunodeficiency virus type 1 is the aetiological agent of AIDS. One of ordinary skill in the art would have had more than sufficient motivation to prepare RT fusion constructs because of the medical importance of this virus and gene product. Utilizing the Salmonella expression system would provide a facile means for generating large quantities of an immunogenic protein that will be capable of inducing both humoral and cell-mediated immune response.

Finality of Office Action

Applicants' amendment necessitated any and all new grounds of rejection. Accordingly, **THIS ACTION IS MADE FINAL.** See M.P.E.P. § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. § 1.136(a). **A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE**

THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 C.F.R. § 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

Correspondence

Any inquiry concerning this communication should be directed to Jeffrey S. Parkin, Ph.D., whose telephone number is (571) 272-0908. The examiner can normally be reached Monday through Thursday from 10:30 AM to 9:00 PM. A message may be left on the examiner's voice mail service. If attempts to reach the examiner are unsuccessful, the examiner's supervisor, James C. Housel, can be reached at (571) 272-0902. Direct general status inquiries to the Technology Center 1600 receptionist at (571) 272-1600. Formal communications may be submitted through the official facsimile number which is (703) 872-9306. Hand-carried formal communications should be directed toward the customer window located in Crystal Plaza Two, 2011 South Clark Place, Arlington, VA. Applicants are directed toward the O.G. Notice for further guidance. 1280 O.G. 681. Informal communications may be submitted to the Examiner's RightFAX account at (571) 273-0908.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Respectfully,

Jeffrey S. Parkin, Ph.D.
Primary Examiner
Art Unit 1648

22 January, 2005

Notice of References Cited	Application/Control No. 09/244,195	Applicant(s)/Patent Under Reexamination KITTO ET AL.	
	Examiner Jeffrey S. Parkin, Ph.D.	Art Unit 1648	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

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	N					
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	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	Thimmig, R. L., et al. August 1993. Human immunodeficiency virus reverse transcriptase. J. Biol. Chem. 268(22):16528-16536.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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E. D

Human Immunodeficiency Virus Reverse Transcriptase

EXPRESSION IN *ESCHERICHIA COLI*, PURIFICATION, AND CHARACTERIZATION OF A FUNCTIONALLY AND STRUCTURALLY ASYMMETRIC DIMERIC POLYMERASE*

(Received for publication, July 16, 1992, and in revised form, April 21, 1993)

Roberta L. Thimmig and Charles S. McHenry

From the Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, B172, Denver, Colorado 80262

Human immunodeficiency virus (HIV) reverse transcriptase isolated from viral particles contains two subunits, p51 and p66. We have produced both subunits in separate *Escherichia coli* strains using expression vectors. Stop codons were placed immediately after the codon for the carboxyl-terminal residue of the mature processed p51 and p66 subunits found in viral particles. Insertion of a methionine in front of the HIV protease cleavage site in the recombinant protein enabled synthesis of both subunits with the natural amino-terminal proline, since *E. coli* methionine aminopeptidase cleaves a Met-Pro amino-terminal linkage. That this occurred to an extent greater than 95% was confirmed by sequencing the purified subunits. Examination of the activities of the individual p51 and p66 subunits on a variety of templates and under solution conditions optimized for each subunit revealed a significant catalytic activity for the natural p51 subunit. This result contrasts to results reported earlier for many recombinant forms without the natural amino and/or carboxyl termini. As expected from earlier work, the optimal homopolymeric template for the p66 subunit was poly(rA). For the p51 subunit, poly(dC) was found to be the optimal template; its activity is 2- to 4-fold greater than p66 on poly(dC). The p51 subunit is 13- to 50-fold less active on poly(rC). These findings are discussed in the context of our earlier hypothesis (McHenry, C. S. (1989) in *Molecular Biology of Chromosome Function* (Adolph, K., ed) Chap. 5, Springer-Verlag, New York) that the HIV reverse transcriptase might be *functionally* asymmetric with distinct plus- and minus-strand polymerases.

Retroviruses contain genomic single-stranded RNA that, upon entry into host cells, is converted to duplex DNA that can integrate into host chromosomes (for reviews, see Gilboa *et al.* (1979) and Weiss *et al.* (1984, 1985)). Since this reaction requires copying RNA into DNA, a reaction with no normal cellular equivalent, retroviruses must encode the necessary enzyme machinery. Retroviral reverse transcriptases copy plus-stranded viral RNA into a complementary minus strand of DNA. The RNA template is then destroyed by an RNase H activity carried within the reverse transcriptase. The free DNA strand is copied to generate a plus strand of DNA resulting in viral duplex DNA that assembles into a nucleoprotein complex capable of integrating into the host genome. Minus-strand DNA synthesis is primed by a cellular tRNA that binds to reverse transcriptase and a complementary sequence within the viral RNA. Plus-strand DNA synthesis is primed by a specific segment of RNA preserved from the destructive activity of RNase H. Thus, the reactions catalyzed by reverse transcriptase are very different. In one reaction, reverse transcriptase must bind a specific tRNA primer and copy an RNA template. In the other reaction, a DNA template is used and RNase H is required to generate the primer.

Human immunodeficiency virus (HIV)[1] is a retrovirus that appears to follow the same rules established for the replication of other retroviruses (for reviews, see McHenry (1989) and Vaishnav and Wong-Staal (1991)). Priming by human $tRNA_3^{Lys}$ for minus-strand DNA synthesis and the specific action of RNase H in generating a specific RNA primer from the polypurine stretch for plus-strand synthesis has been demonstrated (Wain-Hobson *et al.*, 1985; Bordier *et al.*, 1990; Wöhrl and Moelling, 1990; Huber and Richardson, 1990; Pullen and Champoux, 1990; Dontsova *et al.*, 1991; Rhim *et al.*, 1991). HIV encodes a reverse transcriptase homologous to that of other retroviruses (Johnson *et al.*, 1986). HIV reverse transcriptase has been shown to be moderately processive, incorporating 50 to 300 nucleotides per association-catalysis-dissociation event depending on the solution conditions and template used (Huber *et al.*, 1989). The enzyme is capable of facilitating template transfer reactions on homopolymers without dissociating (Huber *et al.*, 1989) and without the need for RNase H activity, at least on homopolymeric templates (Buiser *et al.*, 1991).

The HIV reverse transcriptase is a structurally asymmetric dimer with subunits of 51,000 and 66,000 Da, generated by differential proteolytic processing from a common precursor (Chandra *et al.*, 1986; Barr *et al.*, 1987; Larder *et al.*, 1987; Farmerie *et al.*, 1987; Hansen *et al.*, 1988). A large variety of sources have been used for HIV reverse transcriptase. The recombinant p66 subunit has been generated as a fusion protein and as a near-native protein with small extensions or deletions on the amino and/or carboxyl termini (Tanese *et al.*, 1986; Larder *et al.*, 1987; Hizi *et al.*, 1988; D'Aquila and Summers, 1989; Müller *et al.*, 1989; LeGrice and Grüninger-Leitch, 1990). An approximation of the p51 subunit was produced by proteolysis of p66 by an endogenous *E. coli* protease (Lowe *et al.*, 1988; D'Aquila and Summers, 1989; Deibel *et al.*, 1990). Many recombinant p51 clones terminate 12–13 codons prior to the natural terminus at a convenient *KpnI* restriction endonuclease cleavage site (Larder *et al.*, 1987; Hizi *et al.*, 1988; Müller *et al.*, 1989; Becerra *et al.*, 1991).

* This work was supported by Grant RO1 AI 26600 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: HIV, human immunodeficiency virus; DTT, 1,4-dithiothreitol; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ddTTP, 2′3′-dideoxythymidine 5′-triphosphate.

Many of these clones were constructed before it became known that the p51 carboxyl terminus was generated by cleavage after Phe-440[2] by the HIV protease (Mizrahi *et al.*, 1989; Bathurst *et al.*, 1990; Graves *et al.*, 1990). Nevertheless, these proteins have been useful in characterizing the basic activities of the HIV reverse transcriptase and in discovering many of the fundamental differences between the p66, p51, and the p51·p66 heterodimeric forms.

Natural HIV reverse transcriptase has been produced in moderate levels in yeast by placing termination codons at the sites of proteolytic cleavage, generating precise carboxyl termini that correspond to viral p51 and p66 (Bathurst *et al.*, 1990). The amino termini of these proteins were also authentic. Only a methionine was placed immediately before the natural amino-terminal proline (Lightfoote *et al.*, 1986) in the recombinant enzyme (Bathurst *et al.*, 1990). The yeast methionine aminopeptidase efficiently removed this residue, revealing the authentic amino terminus. Other recombinant expression vectors have been constructed that produce p66 with termini identical with those of p66 isolated from viral particles (Deibel *et al.*, 1990; Becerra *et al.*, 1991). Natural HIV reverse transcriptase has also been produced in *E. coli* by expression of the HIV protease either in *cis* on the same precursor, as in the virus, or in *trans* as a separate protein that can act on a recombinant p66 precursor to generate a p66·p51 heterodimer in *E. coli* (Farmerie *et al.*, 1987; Le Grice *et al.*, 1987; Mizrahi *et al.*, 1989; Hostomsky *et al.*, 1991). This latter approach has been used successfully to generate large quantities of p51·p66 HIV reverse transcriptase indistinguishable from that isolated from viral particles (Mizrahi *et al.*, 1989; Hostomsky *et al.*, 1991).

The p51 and p66 subunits associate very tightly to form a heterodimer ($K_{association} = 5 \times 10^5$ M^{-1}). The p66 subunits associate more weakly ($K_{association} = 2\text{–}5 \times 10^4$ M^{-1}). Stable association of the p51 subunits has not been detected (Restle *et al.*, 1990; Becerra *et al.*, 1991). Only the dimeric forms of reverse transcriptase subunits appear to exhibit significant activity (Rowley *et al.*, 1990; Restle *et al.*, 1990). The p51 subunit by itself has been found to exhibit either no (Hizi *et al.*, 1988; Starnes *et al.*, 1988) or low catalytic activity (Larder *et al.*, 1987). Under solution conditions optimized for p51 (low ionic strength), the highest level of activity observed for the p51 form was 28% of the maximal activity of the p66 subunit (Hostomsky *et al.*, 1991) on a poly(rA) template. Even under these conditions, a dimeric form of p51 was not detectable (Hostomsky *et al.*, 1991).

The carboxyl-terminal domain of the p66 HIV reverse transcriptase contains the RNase H domain (Johnson *et al.*, 1986; Hansen *et al.*, 1988; Prasad and Goff, 1989) which is not present in p51. The isolated p15 RNase H exhibits low activity (Hansen *et al.*, 1988; Hostomsky *et al.*, 1991), but is activated upon binding the p51 subunit, indicating synergistic interactions between these two domains (Hostomsky *et al.*, 1991).

The structural asymmetry of HIV reverse transcriptase raises the possibility of functional asymmetry. A role for dimeric replicative complexes was first proposed for the bacteriophage T4 system in a model to explain the retargeting of the lagging-strand polymerase to a series of Okazaki fragments on the same fork rather than associating with other competing templates in solution (Sinha *et al.*, 1980). A similar dimeric complex was proposed in *E. coli* (Kornberg, 1974). We have extended those findings to include an asymmetric dimeric polymerase with properties expected of a functionally distinguishable leading- and lagging-strand polymerase (McHenry and Johanson, 1984; Johanson and McHenry, 1984; Hawker and McHenry, 1987; McHenry, 1988; McHenry, 1991). It has been proposed that such an enzyme might coordinate action on both sides of the replication fork through allosteric interactions and permit the lagging-strand half to rapidly recycle while keeping the leading-strand half continuously associated with the replication fork. Similar proposals have been made for an asymmetric dimer in eukaryotic systems. Work in SV40 supports the notion of distinct leading- and lagging-strand polymerases, with the δ and α polymerases, respectively, performing these roles (Tsurimoto *et al.*, 1990). More recent models include yet a third polymerase, ϵ, on the lagging-strand (Morrison *et al.*, 1990; Burgers, 1991). Work is underway to identify the link between the leading- and lagging-strand apparatus.

Based on the knowledge that reverse transcriptases must catalyze distinct RNA and DNA template-directed reactions and that the HIV reverse transcriptase is a structurally asymmetric dimer when isolated from viral particles, we proposed that it might also be functionally asymmetric with distinct plus- and minus-strand polymerases (McHenry, 1989). Such functional asymmetry might arise from the structural asymmetry of the enzyme and serve to optimize each activity for a unique role in the retroviral replicative reaction. A definitive test of such a hypothesis requires complete reconstitution of the retroviral reaction and identification of activities relevant in a complete interacting system. As a step toward this goal, we have generated model templates containing RNA sequences of the natural long terminal repeats, synthetic human $tRNA_3^{Lys}$ primers, recombinant HIV nucleocapsid protein that coats the viral RNA template, and individual recombinant p51 and p66 subunits with termini identical with those produced within the virus. In this report, we describe our preliminary characterization of the activity of the HIV reverse transcriptase subunits on model homopolymeric templates that yields results consistent with our functional asymmetry hypothesis.

MATERIALS AND METHODS

Plasmids—pLMRTc (obtained from Drs. Laura Moen and Daniel Santi, University of California, San Francisco) expresses a modified form of p66 lacking 22 residues from the carboxyl terminus. The reverse transcriptase sequences of pLMRTc were obtained from pAB24/RT4 (Barr *et al.*, 1987) where the first 72 nucleotides of the amino-terminal sequence were reconstructed by a synthetic oligonucleotide and a restriction endonuclease site (*Hin*dIII) was introduced. pBBMD11, a derivative of pJF119EH (Fürste *et al.*, 1986; McHenry *et al.*, 1989), contains an IPTG-inducible *tac* promoter and allows translational coupling if needed for production of expressed proteins.

Bacterial Strains—*E. coli* strains used were TG1 (*sup*E, *thi*, *hsd*D5, Δ(*lac-pro*AB) (F′ *tra*D36, *pro*AB, *lac*I^{q}ZΔM15) (Amersham)) and D1245 (*rec*A56, *hsd*S20, *rps*L20, ΔD(*lac*)X74, *pro*A2, *ara*14, *xyl*5, *mtl*1, *sup*E44 (obtained from Dr. Joan Betz of this department)).

Cloning Enzymes—T4 DNA ligase, DNA modifying enzymes, and restriction enzymes were obtained from New England Biolabs or Promega. Standard manipulations of DNA for cloning purposes were followed according to protocols of Sambrook *et al.* (1989). DNA fragments used for cloning were isolated by agarose gel electrophoresis and eluted using an Elutrap (Schleicher and Schuell).

Site-directed Mutagenesis—Mutagenesis was performed with the oligonucleotide-directed *in vitro* mutagenesis system (Amersham) which was based on the method of Eckstein and co-workers (Taylor *et al.*, 1985a, 1985b). Single-stranded DNA was generated by infecting cells with target plasmids that contain the M13 origin with helper phage R408 (Stratagene). The single-stranded DNA template for mutagenesis was recovered from the polyethylene glycol-precipitated phage particles by phenol extraction and ethanol precipitation (Flower and McHenry, 1986; Sambrook *et al.*, 1989). TG1 cells were transformed with the mutated double-stranded DNA and selected for ampicillin resistance. Resulting colonies were screened individually

[2] Amino acid numbers are from the HIV-1 SF2 isolate (Sanchez-Pescador *et al.*, 1985).

for the newly created restriction endonuclease site, and the region of mutation was verified by DNA sequencing.

Polymerase Chain Reaction—The polymerase chain reaction (Mullis and Faloona, 1987) was conducted using the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus Instruments) and the protocols outlined by the manufacturer using a Perkin-Elmer Cetus DNA Thermal Module.

DNA Sequencing—DNA obtained from isolates that had been subjected to site-directed mutagenesis were sequenced by single-stranded dideoxy methods (Sanger *et al.*, 1977) with modifications (Flower and McHenry, 1986). To verify the amino and carboxyl termini-encoding sequences of pRT100A, the plasmid was sequenced by Lark Sequencing (Houston, TX) by dideoxy nucleotide termination reactions using a double-stranded template.

Buffers—These were: Buffer A (50 mM Tris·HCl (pH 7.5), 6% glycerol, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM ethyl *p*-aminobenzoate (Sigma), 0.4 mM phenylmethylsulfonyl fluoride (Sigma)); Buffer B (50 mM 1,3-diaminopropane (pH 8.8), 6% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM ethyl *p*-aminobenzoate, 0.4 mM phenylmethylsulfonyl fluoride); Buffer C (50 mM Tris·HCl (pH 7.5), 6% glycerol, 50 mM NaCl, 0.1% Triton X-100 (Pierce), 1 mM DTT, 1 mM EDTA, 1 mM ethyl *p*-aminobenzoate, 0.4 mM phenylmethylsulfonyl fluoride); and enzyme dilution buffer (50 mM Tris·HCl (pH 7.5), 20% glycerol, 0.1 mM EDTA, 0.2 mg/ml bovine serum albumin).

Chromatographic Supports—DEAE-Sephacel and Q-Sepharose Fast Flow were purchased from Pharmacia LKB Biotechnology Inc. Single-stranded DNA cellulose was obtained from Sigma.

Assays—The RNA template-directed elongation activity of fractions generated during the purification was determined on the primed template (poly(rA)·$(dT)_{12-18}$ (Pharmacia)) at 37 °C in a reaction mixture of 50 μl containing 50 mM Tris·HCl (pH 8.5), 6 mM $MgCl_2$, 80 mM KCl, 8 mM DTT, 0.05% Triton X-100, 100 μM [^{3}H]TTP (specific activity, 200 cpm/pmol TTP (ICN)), and 1 μg of primed template (3 nmol of template nucleotide). When necessary, the enzyme was diluted in enzyme dilution buffer and added last in quantities no larger than 10 μl. The reaction was terminated after 30 min by the addition of 2 drops of 0.2 M sodium pyrophosphate and 0.5 ml of 10% trichloroacetic acid and filtered through Whatman GF/C filters. Filters were washed with 1 M HCl plus 0.2 M sodium pyrophosphate and dried. Liquid scintillation counting was performed with a Beckman LS 3801 using Ecoscint O (National Diagnostics).

Where indicated, other primed templates, appropriate radioactive deoxynucleotide, and salt concentrations were substituted or varied in the reverse transcriptase assay conditions. One unit of reverse transcriptase is defined as the amount that catalyzes the incorporation of 1 pmol of deoxynucleotide in 1 min at 37 °C on a primed template.

Protein concentrations were measured by the Bradford assay (Bio-Rad) using γ-globulin as a standard. Conductivity was determined by a Radiometer CDM2e Conductivity Meter.

Treatment of Reverse Transcriptase—Reverse transcriptase subunit p66 (Fraction V) and reverse transcriptase p51 (Fraction V) were analyzed for reverse transcriptase activity 24–96 h after being thawed. Reconstituted heterodimer was formed by mixing together equal moles of p66 and p51 and allowing the subunits to associate overnight at 4 °C before reverse transcriptase activity was analyzed under the stated conditions.

Nucleic Acids—Primed templates (poly(rA)·$(dT)_{12-18}$, poly(dA)·$(dT)_{12-18}$, poly(rC)·$(dG)_{12-18}$, poly(dC)·$(dG)_{12-18}$), homopolymers (poly(rI) and poly(dI)), and primers (oligo$(dC)_{12-18}$, oligo$(rA)_{12-18}$, and oligo$(dA)_{12-18}$) were from Pharmacia. Homopolymers poly(U) and poly(T) were obtained from Midland Certified Reagent Co. (Midland, TX). All nucleic acids were resuspended to desired concentrations in sterile 10 mM Tris (pH 7.5) and 1 mM EDTA. Equimolar quantities of primers and templates, based on nucleotide, were annealed and added to the assay in the indicated quantity.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Slab gel electrophoresis was conducted in a 7.5–12.5% acrylamide gel (5% stacking gel) in 0.1% SDS as described (Laemmli, 1970; McHenry and Crow, 1979). Protein was detected by Coomassie Blue staining. Densitometry was performed on a Molecular Dynamics Computing Densitometer.

Amino Acid Sequence Analysis—The amino acid sequence of the amino terminus of p51 and p66 was determined by Julie Lippincott (University of Colorado Cancer Center) on an Applied Biosystems 477A Protein Sequencer using the procedures and reagents recommended by the manufacturers.

RESULTS

Construction of Plasmids Used for Production of p66 and p51

Plasmids were constructed to produce either the p51 or the p66 subunit with the same sequence found in reverse transcriptase isolated from viral particles. Both p51 and p66 are generated by cleavage from a common precursor creating proline as the terminal amino acid (Veronese *et al.*, 1986; Lightfoote *et al.*, 1986). Cleavage by the HIV protease after Phe-440 generates p51 and cleavage after Leu-540 generates p66 (Lightfoote *et al.*, 1986; Debouck *et al.*, 1987; Darke *et al.*, 1988; Graves *et al.*, 1988; Mizrahi *et al.*, 1989; Bathurst *et al.*, 1990). To produce these proteins in *E. coli* without using the HIV processing protease, we placed stop codons immediately after the codon corresponding to the proteolytic cleavage site. We exploited the specificity of the *E. coli* methionine aminopeptidase to generate proteins with proline at the amino terminus. This enzyme removes methionine from the ends of proteins containing small amino acids in the penultimate position, and its specificity includes cleavage of Met-Pro bonds (Ben-Bassat *et al.*, 1987; Devlin *et al.*, 1988). These sequences were placed downstream of the strong, inducible *tac* promoter and an optimal *E. coli* translational initiation sequence (Gold and Stormo, 1990).

Site-directed mutagenesis was used to insert a termination codon (TAG) in place of Tyr-441, the residue immediately following the proteolytic processing site of p51 (Fig. 1, *step A*). An adjacent downstream *Sal*I restriction endonuclease cleavage site was introduced simultaneously. The presence of the newly introduced *Sal*I site was confirmed in two isolates (pRT404 and pRT429), and the region of mutation was verified in both isolates by DNA sequencing. Because these plasmids contained a T7 RNA polymerase promoter, the production of p51 was first attempted by induction from this promoter (Studier and Moffatt, 1986; Studier *et al.*, 1990) but without success in obtaining material useful for further purification. Thus, the engineered p51 sequences were placed under the control of a *tac* promoter vector, pBBMD11, used by us previously for the successful overproduction of several other proteins. A synthetic oligonucleotide containing the amino terminus of HIV reverse transcriptase up to codon Lys-11 (*Hind*III site) and a downstream *Sal*I site to facilitate later cloning steps (Fig. 1, *step c*) were inserted into pBBMD11. Where appropriate, the degeneracy of the genetic code was exploited to substitute commonly used *E. coli* codons for those used by HIV. The resulting plasmid, pRT538, was cleaved, and the remaining p51 sequences (*Hind*III to *Sal*I sites of pRT429) were introduced (Fig. 1, *step f*). The resulting plasmid, pRT581, contained all features designed for the production of a protein identical in amino acid sequence with the p51 subunit of HIV reverse transcriptase.

To create a vector suitable for p66 production, the carboxyl-terminal sequences missing from the p51 vector were inserted upstream of the engineered p51 stop codon at a convenient *Hpa*I restriction site (Fig. 1, *step j*). These p66 sequences were generated from an HIV proviral clone by the polymerase chain reaction. One primer upstream of the internal *Hpa*I site was used (see Fig. 1 legend). A second downstream primer that was complementary to the carboxyl terminus of the processed p66 subunit also contained a 5′-noncomplementary region that created a stop codon after the desired Leu-560 carboxyl-terminal residue and a *Sal*I restriction site to facilitate cloning ((Fig. 1, *step g*). The reaction product was cleaved with *Hpa*I and *Sal*I and inserted into cleaved pRT581 to generate the p66 expression vector, pRT100A, used in these studies (Fig. 1, *step j*).

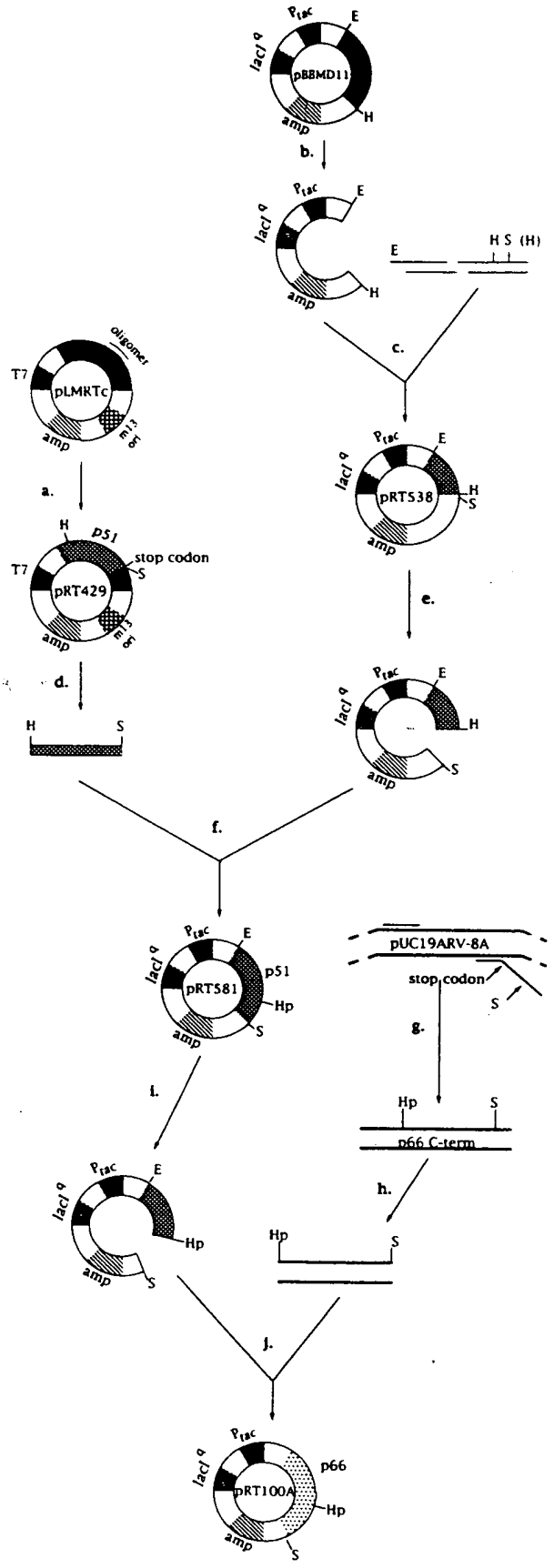

FIG. 1. **Construction of plasmids that express the p51 and p66 subunits of HIV reverse transcriptase.** Plasmids pRT581 and pRT100A were constructed to contain the coding sequences for p51 and p66 reverse transcriptase subunits. They are both transcribed from the *tac* promoter in the presence of IPTG. Both follow a perfect *E. coli* ribosome binding site, AAGAAGU, and contain optimal *E. coli* codons in the initial coding sequences of their mRNA. *Step a*, site-directed mutagenesis of pLMRTc was conducted as described under "Materials and Methods" using the oligonucleotide: 5′-GCA-GAACTTTCTAGGTCGACGGGGCAGCT-3′, which was complementary to the single-stranded M13 DNA template except for the *SalI* (*S*) site (*underlined*) and stop codon (*double underlined*). *Step b*, pBBMD11 was digested with *Eco*RI (*E*) and *Hin*dIII (*H*), and a 5239-base pair fragment was isolated by gel electrophoresis as described under "Materials and Methods." *Step c*, to produce the plasmid pRT538, the following ligated and annealed oligonucleotides were inserted into the pBBMD11 fragment produced in *step b* (Sequence I). pRT538 contained features that were expected to increase the production of p51, including a ribosome binding site 9 bases upstream from an initiating AUG that was placed in front of the proline found at the amino terminus of the mature processed viral reverse transcriptase. Also, an adenylate, 3 residues upstream of the initiating AUG, was imbedded in an exclusively AT-containing region to increase translation (Stormo, 1986). The oligonucleotide also reconstructed the 5′-encoding sequences of p51 up to the first convenient restriction site within the p51 sequences of pRT429 (*Hin*dIII) and provided a *SalI* site for further cloning steps. *Step d*, pRT429 was digested with *Hin*dIII and *SalI*, and a 1293-base pair fragment containing the remaining p51 sequences was isolated. *Steps e* and *f*. The fragment generated in *step d* was inserted into pRT528 which had been digested with *Hin*dIII and *SalI*, resulting in pRT581, the vector used for expression of p51 in these studies. *Step g*, oligonucleotides 5′-CA-GAAGCAGGGGCAAGGCCAATGG-3′ and 5′-CACGACTGCCCG-GGGTCGACTTATAGTACTTTCCTGATTCCAG-CAC-3′ were used to amplify a 691-base fragment from pUC19ARV2-8A (bases 3092–3783) using the polymerase chain reaction. The sequences complementary to ARV2–8A (Sanchez-Pescador *et al.*, 1985) are underlined, the stop codon is double-underlined (not part of complementary sequence), and the *SalI* site is set in bold type. *Step h*, the amplified fragment was cut with *HpaI* and *SalI* (*steps i* and *j*) and ligated into pRT581 that had been digested with the same restriction endonucleases. The new construct, pRT100A, was used to express the p66 subunit.

Purification of p51 Reverse Transcriptase

HIV-1 p51 was purified 22-fold, relative to the ammonium sulfate fraction, from induced *Escherichia coli* cells containing pRT581 (Table I). All operations were performed at 0–4 °C. Enzyme fractions obtained from chromatographic procedures that contained at least 50% of the peak activity were combined.

Cell Growth and Induction—Strain D1245 containing pRT581 was grown at 30 °C in 175 liters of medium containing 2 kg of yeast extract, 1.6 kg of NZ-amine (peptone), 2 kg of $K_2HPO_4 \cdot H_2O$, 0.32 kg of KH_2PO_4, 6.6 g of ampicillin, and 2.4 kg of glucose with high aeration (220 liters/min, 10 psi). The pH was maintained at 7.4. When cells reached midexponential phase (OD_{600} = 0.6), 1 mM IPTG and 6.6 g ampicillin were added. Cells were harvested 5 h after induction in a continuous flow centrifuge after passing through a 75-foot stainless steel coil submerged in ice to cool the cells to 11 °C. Cells were weighed, resuspended in an equal weight of chilled 50 mM Tris·HCl (pH 7.5), 10% sucrose, and immediately poured into liquid N_2. Approximately 1.8 kg of cells were recovered.

Preparation of Extracts D1245/pRT100A—Frozen cells (42 g of cells, 84 g total resuspended weight) were thawed in a solution of 50 mM Tris·HCl (pH 7.5), 10% sucrose, 0.1 M NaCl, 2.5 mM EDTA, 15 mM spermidine, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM ethyl *p*-aminobenzoate (0.2 g of cells/ml, final concentration). The pH was adjusted to 7.6 with 2 M Tris, and egg white lysozyme (0.4 mg/ml, Worthington) was then added. This mixture was transferred to 250-ml centrifuge bottles, incubated at 4 °C for 2 h, and then heated in a 37 °C bath for 10 min with gentle inversion of the bottles each minute. Cells were centrifuged (23,000 × *g*, 1 h) and the supernatant, Fraction I, was recovered (125 ml).

Ammonium Sulfate Fractionation—To each ml of Fraction I was added 0.314 g of $(NH_4)_2SO_4$. After dissolving $(NH_4)_2SO_4$,

SEQUENCE I

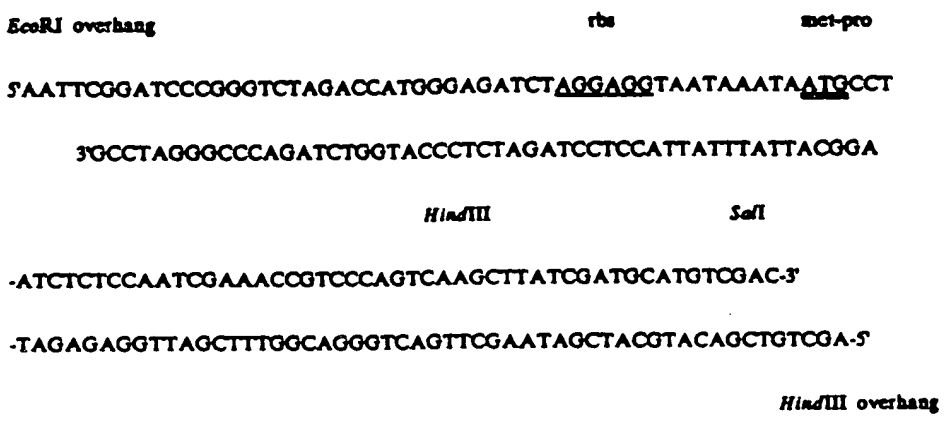

TABLE I

Purification of HIV p66 and p51 from E. coli

Purification of p66 and p51 was carried out on 25 g and 42 g of cells, respectively.

Fraction	p66			p51		
	Total protein	Total units	Specific activity	Total protein	Total units	Specific activity
	mg	$\times 10^{-6}$	$\times 10^{-3}$ *units/mg*	*mg*	$\times 10^{-6}$	$\times 10^{-3}$ *units/mg*
I. Lysate supernatant	1548	9.6	6.2	2336	ND[a]	ND[a]
II. Ammonium sulfate						
Precipitate	509	12.5	24.4	1572	7.1	4.5
Dialysate	453	11.7	25.9	1755	6.3	3.6
III. DEAE-Sephacel	75	8.2	109.4	319	6.9	21.7
IV. Q-Sepharose	32	4.1	129.8	—[b]	—[b]	—[b]
V. Single-stranded DNA cellulose	6	1.3	204.0	43	4.2	98.4

[a] Reverse transcriptase assay results for p51 in Fraction I were not reliable (nonlinear) and are, therefore, not reported. ND, not determined.
[b] Purification of p51 did not include a Q-Sepharose column.

the suspension was placed on ice for 1 h. The precipitate was recovered by centrifugation (23,000 × *g*, 30 min) and was stored at −70 °C until needed. The pellet was resuspended in 50 ml of Buffer A and dialyzed *versus* 1 liter of Buffer A overnight to yield Fraction II (54 ml).

DEAE-Sephacel—This procedure is a modification of that of Müller *et al.* (1989). Dialyzed Fraction II was applied to a DEAE-Sephacel column (2.5 cm × 6.2 cm) equilibrated in Buffer A (1 column volume/h). The column was washed with 2 column volumes. Reverse transcriptase activity was recovered in the flow-through fractions (Fraction III, 57 ml).

Single-stranded DNA Cellulose—Fraction III was applied to a single-stranded DNA cellulose column (2.5 cm × 4 cm) equilibrated in Buffer C at 1 column volume/h, and the column was washed with 2 column volumes. The column was developed with a 10-column volume linear gradient of 50–800 mM NaCl. The majority of protein flowed through the column, whereas the reverse transcriptase activity of p51 was recovered as a single symmetrical peak at 180 mM NaCl (Fraction V, 18 ml, Fig. 2). The purified enzyme fractions were divided into aliquots and rapidly frozen in liquid nitrogen and stored at −70 °C.

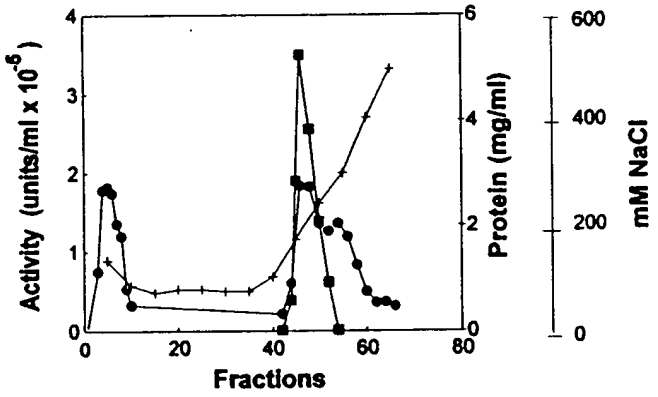

FIG. 2. **Chromatography of the p51 subunit of HIV-1 reverse transcriptase on single-stranded DNA cellulose.** Fraction III was applied to a single-stranded DNA cellulose column. The column was washed and developed with a 50–800 mM NaCl linear gradient. Reverse transcriptase activity (●), protein (○), and conductivity (■) were analyzed as described under "Materials and Methods."

Purification of p66 Reverse Transcriptase

The p66 subunit of reverse transcriptase was purified 33-fold relative to Fraction I by a procedure identical with that used for p51 except for the following modifications. Strain D1245/pRT100A was grown to midlog phase (OD_{600} = 1.0) and induced for 3 h, yielding 0.7 kg of cells. Extracts of frozen cells (25 g cell weight, 50 g total) were prepared, yielding 98 ml recovered as Fraction I (Table I). The p66 reverse transcriptase activity was found in the flow-through fraction of the DEAE-Sephacel column (Fraction III, 25.5 ml). The pH of Fraction III of p66 was increased to 8.5 gradually by the addition of 1 M 1,3-diaminopropane and then chromatographed on a Q-Sepharose column (2.5 cm × 3 cm) equilibrated in Buffer B at 1 column volume/h. The column was washed with 2 column volumes of Buffer B, and the reverse transcriptase activity of p66 was found in the flow-through fraction (Fraction IV, 22 ml). The p66 subunit was recovered as a single peak on a single-stranded DNA cellulose column, eluting at 220 mM NaCl when developed with the same salt gradient used for p51 (Fraction V, 16.5 ml).

Physical Characterization

SDS-Polyacrylamide Gel Electrophoresis—Samples of Fraction V from p51 and p66 were denatured and subjected to

electrophoresis on a 7.5–12.5% gradient SDS-polyacrylamide gel (Fig. 3). The purified p51 subunit migrated with an apparent molecular weight of 54,000. A densitometric scan indicated p51 to be greater than 93% pure. The p51 subunit was recognized by a monoclonal antibody directed against the HIV-1 reverse transcriptase (data not shown). Purified p66 migrated with an apparent molecular weight of 68,000 (Fig. 3*B*) and a densitometric scan indicated that the protein composed 36% of the fraction. An assumed proteolytic product of p66 migrating at 54,000 Da composed 13% of the total p66 fraction, and a protein that migrated with an apparent molecular weight of 61,000 made up 8% of total protein. All three of these proteins were recognized by antibody directed against HIV-1 reverse transcriptase. The higher purity of the p51 subunit reflects its elution at a lower salt concentration from the single-stranded DNA cellulose column compared to p66, separating p51 from contaminants that chromatograph with p66. The two protein contaminants that co-purify with p66 and react with the antibody directed against the HIV-1 reverse transcriptase must share common sequences and chromatographic characteristics of the authentic p66 subunit. The lower molecular weight contaminant migrating with an apparent molecular weight of 15,000 is lysozyme that was added for cell lysis. It was identified by isolation from an acrylamide gel, and the first 9 residues were sequenced. Lysozyme can be removed by the procedure reported in the legend to Fig. 7. The properties of lysozyme-free p66 are indistinguishable from Fraction V. Addition of lysozyme to p66 does not affect its properties.

Amino-terminal Sequencing of p51 and p66—Fraction V of p51 and p66 were resolved on SDS-polyacrylamide gels, transferred to Applied Biosystems ProBlott membranes, and excised from the membrane for amino-terminal sequencing. Greater than 95% of the p51 or p66 protein had proline as the amino terminus (Fig. 4). As predicted, the methionine aminopeptidase of *E. coli* cleaved the Met-Pro peptide bond of the produced proteins, creating proteins with amino termini identical with the p51 and p66 found in the viral particle. The 61,000-Da contaminant in Fraction V of p66 was also sequenced and was found to have an amino terminus (Met-16) identical with the authentic p66, suggesting that it results from internal translational initiation.

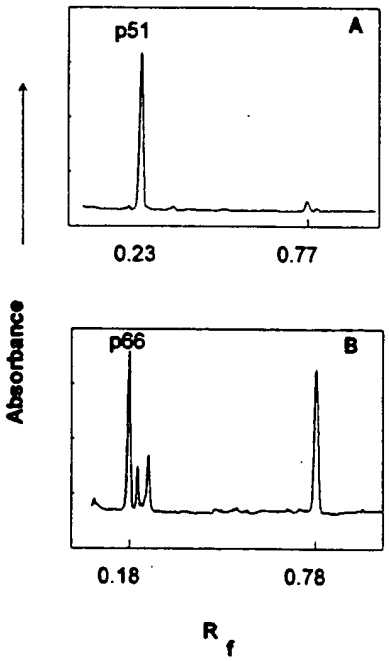

FIG. 3. **Densitometric scans of the purified p51 and p66 subunits resolved by SDS-polyacrylamide gel electrophoresis.** Final fractions of p51 and p66 were subjected to electrophoresis on a 7.5–12.5% SDS-polyacrylamide gradient gel as described under "Materials and Methods." The relative purity of the fractions was determined using a Molecular Dynamics Computing Densitometer. The reverse transcriptase subunits are labeled in *A* and *B*. The contaminant in the p66 fraction has an apparent molecular weight of 15,000.

Predicted sequence from DNA	**Met-Pro-Ile-Ser-Pro-Ile-Glu**
Sequence from purified p51 and p66	**Pro-Ile-Ser-Pro-Ile-Glu**

FIG. 4. **Analysis of amino terminus of p51 and p66.** The protein sequences of p51 and p66 were determined as described under "Materials and Methods" verifying that the methionine aminopeptidase of *E. coli* cleaved the methionine from the produced p51 and p66 leaving the authentic amino terminus.

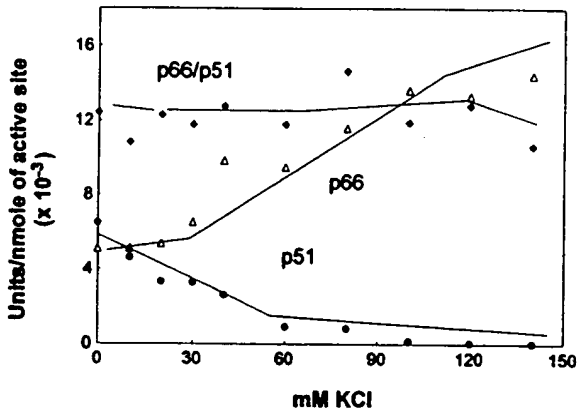

FIG. 5. **Salt sensitivity of p66, p51, and p51·p66 heterodimer.** The polymerase activities of p66 (Δ), p51 (●), and reconstituted heterodimer p66·p51 (♦) were determined in the presence of increasing KCl. Poly(rA)-oligo(dT)$_{12-18}$ was used as template. Incubations were performed at 37 °C for 30 min.

Characterization of Reverse Transcriptase Activity

Availability of the two subunits of reverse transcriptase allowed separate comparison of their activities and of the activity of a reconstituted heterodimer.

Activity of p51, p66, and Reconstituted Heterodimer on Poly(rA)-dT$_{12-18}$ Primed Template in the Presence of Varying Concentrations of KCl—Reconstituted heterodimer, the p51 subunit, and the p66 subunit showed striking differences in reverse transcriptase activity on a poly(rA)·(dT)$_{12-18}$ primed template, depending on the concentration of KCl used in the reverse transcriptase assay (Fig. 5). The p51 subunit showed an 8-fold decrease in activity, whereas the p66 subunit exhibited a 2-fold increase in activity over a 0–60 mM increase in KCl concentration. The reconstituted heterodimer showed no salt sensitivity over the KCl concentration range tested.

The p51 subunit has been reported to have substantially lower activity than p66 (Tisdale *et al.*, 1988; Starnes *et al.*, 1988; Restle *et al.*, 1990). Most published assays for HIV-1 reverse transcriptase have been performed in 80 mM KCl where the p51 subunit is relatively inactive compared with p66. Contrary to these findings, p51 is equal in activity to p66, in assays containing less than 10 mM KCl.

Upon forming the reconstituted heterodimer, not only is a salt-resistant enzyme generated but a synergistic increase in activity is observed. The heterodimer activity is twice what would be expected if p51 and p66 were acting on the template

independently in the mixture. The p66 or p51 subunits were introduced to $(NH_4)_2SO_4$ or $(NH_4)_2HPO_4$, so that the final concentration of salt was 1 M, kept at 4 °C, and assayed after 24 h. The reverse transcriptase activity of the p66 subunit in either ammonium salt was stimulated 2-fold compared with p66 not mixed with the ammonium salts, confirming the results of Rowley *et al.* (1990). The reverse transcriptase activity of p51 mixed with the ammonium salts was decreased (data not shown). The final concentration of ammonium salts in the reverse transcriptase assay was less than 5 mM.

Template Specificity of p51, p66, and Reconstituted p51·p66 Heterodimer—The activity of the three forms of reverse transcriptase on various primed homopolymers at two KCl concentrations was investigated. On a poly(rA) template at the standard 80 mM KCl, p66 demonstrates an 8- to 11-fold greater activity than p51 (Table II, Fig. 6). Switching to a poly(dA) template resulted in a drastic decrease in activities for both enzyme forms. The inability of poly(dA) to act as a suitable template for p66 has been reported by several laboratories (Majumdar *et al.*, 1988; Goff, 1990; Clark *et al.*, 1990; Williams *et al.*, 1990). Our results extend this observation to p51. Changing the template from the standard poly(A) template to poly(C) causes a severe decrease in p51 activity and a lesser decrease for p66. However, use of a poly(dC) template instead of a poly(rA) template resulted in a 5-fold increase of p51 activity in 80 mM KCl. The activity of p51 was 2- to 4-fold greater than that of p66 on a poly(dC) template (Table II, Fig. 6). Interestingly, p51, which shows marked salt sensitivity on poly(A), is insensitive to salt on a poly(dC) template, indicating that the salt effects are not due to simple electrostatic interactions between the polymerase and the phosphodiester backbone. Other templates showed varying levels of low activity. Poly(U), poly(T), and poly(dI) are essentially inert with either form of the enzyme.

TABLE II

Salt sensitivity of enzymes on various templates

The activity of p66, p51, and reconstituted heterodimer p66:p51 was determined on various homopolymer templates primed with various oligomers in the presence of 10 and 80 mM KCl as described under "Materials and Methods." All activities (units/nmol of active site) values were normalized relative to the activity of p66 at 80 mM KCl.

Template	10 mM KCl			80 mM KCl		
	p66	p51	p66·p51	p66	p51	p66·p51
			%			
Poly(rA)·oligo(dT)	45	60	83	100	9	71
Poly(dA)·oligo(dT)	3	<1	2	<1	<1	<1
Poly(rC)·oligo(dG)	11	4	12	19	<1	16
Poly(dC)·oligo(dG)	12	52	28	24	46	37
Poly(rI)·oligo(dC)	5	9	9	14	9	17
Poly(dI)·oligo(dC)	1	<1	1	1	<1	<1
Poly(U)·oligo(rA)	2	<1	<1	<1	<1	<1
Poly(T)·oligo(rA)	<1	<1	<1	<1	<1	<1
Poly(U)·oligo(dA)	8	<1	6	<1	<1	<1
Poly(T)·oligo(dA)	2	<1	4	<1	<1	<1

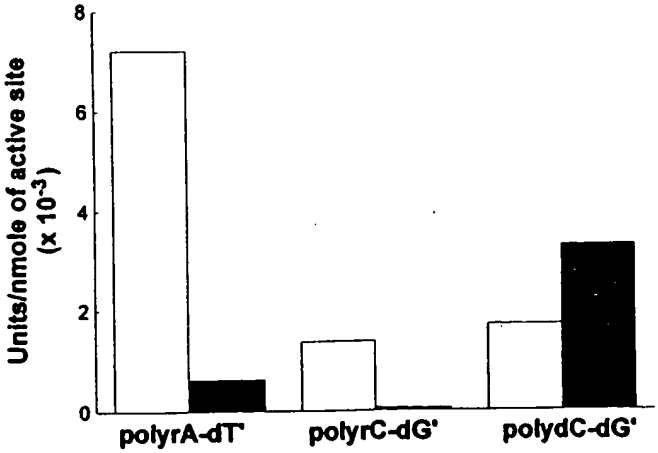

FIG. 6. **Comparison of p66 and p51 polymerase activities on poly(rA)·$(dT)_{12-18}$, poly(dC)·$(dG)_{12-18}$, and poly(rC)·$(dG)_{12-18}$ templates.** The subunits of the reverse transcriptase were assayed at 80 mM KCl at 37 °C for 30 min using 1 μg (3 nmol of total nucleotide) of each template. p66 activity is represented by the *open*

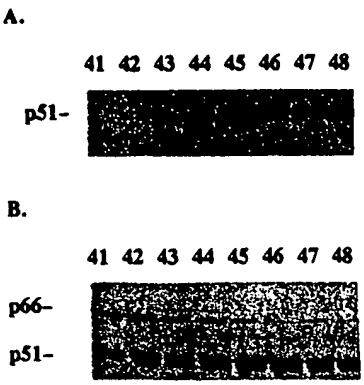

FIG. 7. **Heterodimer formation between p51 and p66.** p66 and p51 (Fraction V) were concentrated separately using Centricon 10 microconcentrators (Amicon) to 40 and 80 μM, respectively. p66 and p51 were mixed to a final concentration of 20 μM and 40 μM, respectively. As controls, experiments with 20 μM p66 and 40 μM p51 were performed separately. A Superose 12 fast protein liquid chromatography column (Pharmacia) was equilibrated in 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, and 0.1% Triton X-100 at 0.1 ml/min with a pressure of 0.75 MPa. Fifty μl of each solution was injected onto an equilibrated column, and 0.3-ml fractions were collected; 20 μl of each fraction was analyzed on a 7.5%-15% polyacrylamide-SDS gradient gel and silver-stained. *A*, Fractions 41–48 of gel filtration chromatography of p51 alone. *B*, Fractions 41–48 of gel filtration chromatography of the mixture of p51 and p66. Locations of p51 and p66 are indicated.

The reconstituted heterodimer mirrored the primed template preference of the p66 subunit (poly(rA)·$(dT)_{12-18}$ > poly(dC)·$(dG)_{12-18}$ > poly(rC)·$(dG)_{12-18}$ >> poly(dA)·$(dT)_{12-18}$) at both salt concentrations and reflected template preference seen by other investigators (Sallafranque-Andreola *et al.*, 1989; Clark *et al.*, 1990).

Association of p51 and p66

The preferential association of p51 and p66 to form a physically asymmetric heterodimer is well-documented (Restle *et al.*, 1990; Becerra *et al.*, 1991). p66 also forms a homodimer, but with a higher dissociation constant; dimers of p51 have not been observed. To verify the ability of our recombinant subunits to form a heterodimer, we mixed the two subunits and demonstrated a shift of p51 to a higher native molecular weight because of its association with p66 (Fig. 7). Alone, p51 eluted as a monomer (peak fraction 45, Fig. 7*A*). When mixed with p66, a second peak of p51 was observed (fraction 42, Fig. 7*B*) at the position consistent with a heterodimer. Subunits p51 and p66 are in roughly[3] an equimolar ratio in this higher molecular weight peak.

DISCUSSION

As a step toward evaluating the contribution the HIV reverse transcriptase p51 subunit makes to the asymmetric

[3] W. Sud silver staining to be variable and only semi-quantitative

dimer generated when it binds the p66 subunit, we have created vectors capable of producing both p66 and p51 in *E. coli* with termini and sequences identical with those isolated from viral particles. Such vectors are critical to future studies using a fully reconstituted HIV replicative system. To avoid perturbing any subtle protein-protein interactions that might be required in the complete system, even if these interactions are unimportant for basic assessment of activity on homopolymeric templates, and to verify that the often-reported low activity of the p51 subunit was not the result of the 12–13 amino acid deletions present in many of the recombinant proteins that are called p51, we placed stop codons immediately after the sites of proteolytic processing that create the p66 and p51 subunits. The amino-terminal proline could not be created by such a simple approach since all proteins must be initiated with methionine. Thus, we exploited the activity of the *E. coli* methionine aminopeptidase which removes methionine when it is followed by proline. Indeed, amino-terminal sequencing of the purified p51 and p66 subunits confirmed that greater than 95% of the residues released in the first cycle were proline. The p51 subunit purified by our procedures was essentially pure. However, our current p66 preparation contained some significant contaminants that cross-reacted with anti-HIV reverse transcriptase serum. Amino-terminal sequencing of one of the contaminants (61 kDa) indicated an internal translational initiation at Met-16. Future efforts will focus on refining the purification methods to avoid these contaminants or on exploiting the degeneracy of the genetic code to weaken the internal bacterial ribosome binding site while preserving the natural reverse transcriptase sequence.

Most of the studies that have found p51 to be inactive have been performed under solution conditions optimized for p66. Our analysis of the activity of p51 on a homopolymeric template, poly(A), which is commonly used for p66 assays confirmed the results of many other investigators that p51 is much less active than p66 (10% in this study) when assayed at 80 mM KCl. Decreasing the salt concentration reduced the activity of p66 and increased the activity of p51 to a point that exceeded p66 activity, and it attained a catalytic efficiency 60% of the optimal p66 level. The salt sensitivity of p51 has been observed previously by Hostomsky *et al.* (1991) in comparison with the p51·p66 heterodimer. Analysis of other templates under a variety of solution conditions indicated that p51 was very inactive on poly(rC) templates. Substituting deoxyribose for ribose led to a greater than 10-fold activation of p51 to a level 2- to 4-fold greater than p66, even in 80 mM salt. Decreasing the salt concentration on this template did not lead to significant further activation of p51, indicating that the salt effect is not general and not due to simple electrostatic interactions between the polymerase and the phosphodiester backbone of the template. Use of other templates revealed varying levels of activity for both enzymes. Particularly surprising was the low level of activity seen on many homopolymeric templates, particularly poly(U), poly(T), and poly(dI). Low levels of activity of p66 had been previously reported on poly(dA) both in homopolymers and oligo(dA) stretches within natural DNA (Majumdar *et al.*, 1988; Goff, 1990; Clark *et al.*, 1990; Williams *et al.*, 1990). Our findings extend this observation to the p51 subunit. These effects are presumably not due to secondary structures, since poly(dT) and poly(U) do not form significant secondary structures in solution. Furthermore, these low activities raise the question of how reverse transcriptase manages to complete the replicative reaction *in vivo*. Overcoming these barriers may require additional host or viral factors.

Based on analogy to other systems, we proposed earlier that one possible role for the p51 subunit might be to combine with p66 to form not only a structurally asymmetric dimeric reverse transcriptase, but also a functional one, with a distinct role for each enzyme in specific stages of retroviral replication. One of the simplest examples of specialized roles would be for a distinct minus-strand (RNA-directed) and plus-strand (DNA-directed) polymerase. If such a mechanism is operative, our results using the poly(dC) template suggest that p66 would serve as the minus-strand RNA-directed polymerase and p51 would serve as the plus-strand DNA-directed polymerase. However, the low activities on homopolymeric templates other than poly(dC) prevents a more general test of this hypothesis, which must be made in the fully reconstituted natural replicative reaction so that proviral DNA can be produced at the rates and specificities expected from *in vivo* studies.

Studies indicate that p66 contributes nearly all of the activity in the p51·p66 heterodimer since mutant p66 drastically diminished the activity of the heterodimer while the same active site mutation in p51 had no effect (Le Grice *et al.*, 1991). However, those assays were conducted on a poly(rA) template under solution conditions optimized for p66, and, in the light of our work, such studies should be extended to poly(dC), although again, more rigorous testing awaits a fully reconstituted reaction. For example, the two halves of the asymmetric dimeric DNA polymerase III holoenzyme interact through allosteric interactions (McHenry *et al.*, 1986, 1988; McHenry, 1988). An analogous scenario for reverse transcriptases would require that the RNA-directed (minus-strand) half be actively engaged in its reaction to elicit a conformational change that activates the DNA-directed (plus-strand) half. Such an interaction is consistent with the role of the RNA-directed minus-strand in initiating the retroviral replication reaction. Maintaining the plus-strand half in an inactive state until the reaction is initiated might prevent unfavorable interactions. Such an effect could complicate homopolymer studies since p66 would have to be activated before any activity could be observed for p51. From the *E. coli* example of an asymmetric dimer, it has become clear that allosteric interactions with other proteins at the fork are also important in establishing all levels of communication for a physiologically relevant coordinated reaction (Wu *et al.*, 1991a, 1991b). Our goal is to develop such a system containing all of the known factors of the retroviral reaction and to develop assays for any missing components.

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Optimization of live oral *Salmonella*–HIV-1 vaccine vectors for the induction of HIV-specific mucosal and systemic immune responses

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Abstract

Recent evidence suggests that live oral *Salmonella*–HIV vaccine vectors have the potential to elicit HIV-specific T cell-mediated immunity in both the mucosal and systemic compartments. We are using the mouse–typhoid model to identify *Salmonella*::HIV vaccine vector constructs that elicit HIV-specific mucosal and systemic immune responses. Oral immunization of mice with a *Salmonella* strain that expresses recombinant gp120 (rgp120) in the cytoplasm of the vector elicits a modest gp120-specific T cell proliferation response in the spleen. However, such *Salmonella* constructs did not stimulate the development of gp120-specific serum IgG or cytotoxic T lymphocytes (CTLs). Interestingly, the majority of cytoplasmically-expressed rgp120 forms inclusion bodies in *Salmonella*. We believe that in this form rgp120 is highly susceptible to protease degradation by the vector. As such, cytoplasmic rgp120 may not persist in the host after vaccination, resulting in the modest immunogenicity of rgp120 in these constructs. To circumvent this problem we constructed *Salmonella* strains that express rgp120 on the surface of the vector. Preliminary data suggest that surface-expressed rgp120 is significantly more immunogenic in both the mucosal and systemic compartments than cytoplasmic rgp120. These results, therefore, support the proposal that *Salmonella* vectors will be a safe and inexpensive means for delivery of HIV antigens to, and the elicitation of HIV-specific T cells in, the mucosal and systemic compartments.

Keywords: *Salmonella*; Vaccine vector; Mucosal immunity; HIV-1; gp120

1. Introduction

Although correlates of protective immunity to HIV-1 in humans have not been fully elucidated, it is

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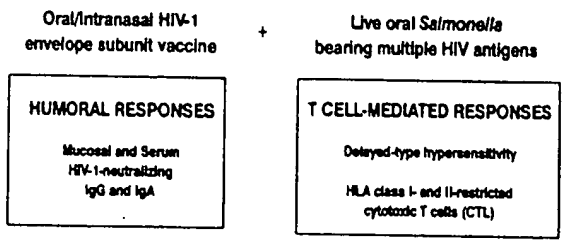

Fig. 1. A prototype combination HIV-1 vaccine. We propose that combination HIV-1 vaccines should consist of a component that elicits broadly neutralizing mucosal and serum antibody, and a second component that elicits broad HIV-1-specific HLA class I- and class II-restricted T cell-mediated responses in both the mucosal and systemic compartments.

becoming clear that an effective HIV-1 vaccine should stimulate broadly neutralizing antibody and HLA class I- and class II-restricted T cell-mediated responses in the mucosal and systemic compartments (Berman et al., 1991; Mackewicz et al., 1991; Koup and Ho, 1994). One approach to elicit such immunity is to develop a combination vaccine which comprises a component that optimally induces HIV-1 envelope-specific neutralizing mucosal and systemic antibody, and a component that induces potent HIV-1-specific mucosal and systemic $CD4^+$ and $CD8^+$ T cell-mediated responses (Fig. 1). We believe that attenuated live oral *Salmonella* vaccine vectors are an attractive tool to serve as the latter component (Fouts et al., 1993). In support, live oral *Salmonella* vaccine vectors have been shown to elicit $CD4^+$ and $CD8^+$ T cell responses in laboratory animals (Yang et al., 1990; Gao et al., 1992; Turner et al., 1993; Aggarwal et al., 1990). The long-term objective of our group, therefore, is to optimize the capacity of our live oral *Salmonella*–HIV vaccine vectors to elicit HIV-specific HLA class I- and class II-restricted T cell immunity against multiple HIV antigens, in the mucosal and systemic compartments. As a first step toward our long-term goal, we are using the mouse–typhoid model to identify *Salmonella*::HIV vaccine vector constructs that elicit host mucosal and systemic immune responses.

2. Results

2.1. Construction of a cytoplasmic gp120 expression cassette

Using standard recombinant DNA techniques (Sambrook et al., 1989) we constructed a cytoplasmic expression cassette that encodes rgp120 under the control of constitutive promoter $P_{lpp/lacUV5}$ (Fouts et al., 1993, 1995a). When mice were immunized orally with a *Salmonella aro* mutant carrying a single copy of this expression cassette integrated into the chromosome of the vector (rgp120 accumulates to a level of 0.05–0.1% of the total protein in this construct), no significant humoral or cellular immune responses were observed. In contrast, mice immunized orally with a *Salmonella aro* mutant carrying multiple copies of the rgp120-expression cassette (rgp120 accumulates to a level of 1–5% of the total protein in this construct) developed measurable gp120-specific IFN-γ secretion and T cell proliferative responses in the spleen (Fouts et al., 1995a, b). This observation suggests that *Salmonella* vectors must produce extremely high levels of cytoplasmic rgp120 to induce host immunity against this recombinant protein.

The folding pattern of cytoplasmically-expressed rgp120 in *Salmonella* was extensively analyzed using a series of monoclonal antibodies that recognize exposed and unexposed continuous epitopes along gp120 (Fouts et al., 1995a). This analysis revealed that expression of rgp120 in the cytoplasm of *Salmonella* produces a denatured product. For example, the V3 loop in native gp120 is surface-exposed on native baculovirus- and CHO cell-produced gp120 but upon heat-denaturation of this glycoprotein the V3 loop becomes embedded within gp120. In contrast, the V3 loop is concealed within native *Salmonella*-expressed rgp120 (Fouts et al., 1995a). In addition, we found that rgp120 forms inclusion bodies in *Salmonella* (Fouts et al., 1995a). Inclusion body formation by eukaryotic proteins in prokaryotic systems is common (Schein, 1989; Marston, 1986; King and Haase-Pettingell, 1988; Derman et al., 1993; Goldberg and Golf, 1986) and is thought to be caused by misfolding of foreign polypeptide sequences (Schein, 1989; Marston, 1986). Factors that might cause misfolding of rgp120 include the inabil-

ity of bacteria to form disulfide bonds in the cytoplasm (Derman et al., 1993) and the absence of glycosylation in bacterial expression systems (Schein, 1989; Marston, 1986; King and Haase-Pettingell, 1988).

2.2. Construction of OmpA::tgp120 fusion expression cassettes

We performed an immunoblot analysis of serum IgG taken from before and after immunization of volunteers with live oral *Salmonella* vaccines (Hone et al., 1992). This analysis, which measures the specificity and relative level of serum IgG to continuous epitopes in *Salmonella*, indicated that the majority of *Salmonella*-specific serum IgG is directed against membrane-associated proteins (R. Hall and D. Hone, unpublished observation). Similar observations have been made using sera from *Salmonella*-immune mice (Brown and Hormaeche, 1989). Interestingly, OmpA is consistently among the most immunogenic proteins of *Salmonella* in humans and mice (Brown and Hormaeche, 1989; R. Hall and D. Hone, unpublished observation). Moreover, there are data showing that immunization with live *Salmonella* elicits cell-mediated responses against outer membrane proteins (Udhayakumar and Muthukkaruppan, 1987). Together, this indirect evidence suggests that secretion of rgp120 fused to OmpA in our *Salmonella* vaccine vectors will enhance the immunogenicity of rgp120.

Using PCR, we constructed a low copy expression cassette encoding a chimeric protein consisting of OmpA fused to most of gp120, called OmpA::tgp120 (Wu et al., in press). Analysis of OmpA::tgp120 by immunoblot of SDS-PAGE separated cellular fractions reveals that this fusion protein is located in the Triton X-100-insoluble fraction of the vector membrane and hence is probably in the outer membrane (Wu et al., in press; Nikaido and Vaara, 1984). The molecular mass of the largest product produced by expression of *ompA::tgp120* is ~ 70 kDa, which is the predicted molecular weight of the chimeric OmpA::tgp120 fusion protein (Wu et al., in press). Further, immunoblot analysis showed that the 70 kDa protein is recognized by both rabbit OmpA-specific polyclonal antiserum and anti-gp120 Mabs (Wu et al., in press).

Table 1
HIV-1 gp120-specific responses after oral immunization of mice with *Salmonella*::HIV vaccine vector constructs

Strain	Lamina propria IgA ASC [a]	Serum IgG [b]
H650	< 2	< 0.048
H651 (OmpA::tgp120)	427 ± 111	0.164 ± 0.016
H648 (rgp120)	115 ± 14	< 0.048

[a] gp120-specific IgA ASC were enumerated by ELISPOT (Beagley et al., 1988) using lamina propria lymphocytes (Hornquist et al., 1993). The results are expressed as the number of gp120-specific IgA ASC per 10^4 IgA ASC.

[b] gp120-specific serum IgG levels were measured by gp120-capture ELISA using baculovirus-produced gp120 as antigen (Abacioglu et al., 1994). ELISAs were performed with pooled sera collected from groups of five mice before and 30 days after oral vaccination and diluted 1:200 in normal saline. The results are expressed as mean net increase in optical density ($OD_{490-650}$) ± the standard deviation.

2.3. Immunogenicity of OmpA::tgp120

In a preliminary study, groups of five mice were immunized orally with *Salmonella aro* mutant strains carrying either a cytoplasmic rgp120 expression cassette or an *ompA* expression cassette or an *ompA*::tgp120 expression cassette. The mice were sacrificed 4 weeks after primary immunization and the number of gp120-specific IgA antibody-secreting cells (ASC) in the lamina propria (LP) were enumerated by the ELISPOT assay as described (Beagley et al., 1988). Mice that receive vector controls do not develop significant gp120-specific IgA ASC in the LP. However, our *Salmonella* vector expressing OmpA::tgp120 elicited high levels of gp120-specific IgA ASC in the LP (Table 1). In contrast, multicopy construct expressing cytoplasmic rgp120 induced less gp120-specific IgA ASC (Table 1). Serum antibody was collected from mice before and on day 30 after oral immunization with *Salmonella* alone, *Salmonella* bearing cytoplasmic rgp120 or OmpA::tgp120. Using a gp120-capture ELISA (Abacioglu et al., 1994), we found that only the mice immunized with the OmpA::tgp120 construct developed significant serum IgG to gp120 (Table 1).

3. Discussion

The data presented here show that oral immunization of mice with a *Salmonella* construct bearing

cytoplasmic rgp120 induces a local humoral immune response against gp120. In contrast, a *Salmonella* construct bearing periplasmic tgp120 induces both a local and systemic humoral immune response against gp120.

An explanation why placement of gp120 in *Salmonella* influences the humoral immune response to this antigen by the murine immune system was not directly addressed in this study. Others have shown that cytoplasmically-expressed foreign antigens in *Salmonella* are highly immunogenic. Further, the in vitro expression level of rgp120 is about 5–10-fold more than tgp120 in *Salmonella* (data not shown). In light of these observations, the contrasting immunogenicity of periplasmic tgp120 and cytoplasmic rgp120 probably reflects a qualitative difference in these antigens. Indeed, cytoplasmically-expressed rgp120 forms inclusion bodies (Fouts et al., 1995a, b), which display a decreased half-life in bacteria (Goldberg and Goff, 1986). We believe that this property results in poor in vivo stability of cytoplasmic rgp120 and the failure of this antigen to induce serum IgG responses to gp120. The periplasmic OmpA::tgp120 fusion proteins, on the other hand, may be more stable in vivo, resulting in the induction of both local and systemic antibody against gp120.

Since our long-term goal is to optimize *Salmonella* vector-induced T cell responses, we are currently investigating whether placement of rgp120 in *Salmonella* also influences the magnitude of gp120-specific $CD4^+$ and $CD8^+$ T cell responses in mice. Presently, there is no consensus on the vector configuration that optimizes the ability of *Salmonella* to induce foreign antigen-specific cytotoxic $CD8^+$ CTLs in vivo (Gao et al., 1992; Turner et al., 1993; Aggarwal et al., 1990). Studies by Pfeifer et al. (1993), using an in vitro antigen presentation model and ovalbumin-expressing vaccine vectors, proposed that cellular placement of antigens in the bacterial vector did not influence ability to present in the context of MHC class I molecules. However, since this in vitro system incorporated *Listeria*-elicited peritoneal activated macrophages, we do not know if this model correlates with the specific in vivo inductive events that generate antigen-specific $CD8^+$ CTLs by *Salmonella* vectors.

Antigen-specific $CD8^+$ CTLs were not detected after immunization with recombinant *Salmonella* vaccine vectors that expressed cytoplasmic influenza A nucleoprotein (Gao et al., 1992). This latter study pointed to the possibility that antigen solubility and/or location might influence the induction of foreign antigen-specific $CD8^+$ CTLs by *Salmonella* (Gao et al., 1992). In agreement, we found that cytoplasmic rgp120 forms inclusion bodies and does not induce detectable gp120-specific $CD8^+$ CTLs in mice after oral or parenteral immunization. Collectively, these observations suggest that individual foreign antigen characteristics will dictate the *Salmonella* vector configuration that optimizes the level of such responses. It is reasonable to propose, therefore, that *Salmonella* bearing surface-expressed rgp120 will elicit gp120-specific $CD8^+$ CTLs.

In summary, the results presented here suggest that HIV-specific immunity will be elicited by *Salmonella*::HIV in the mucosal and systemic compartments. Currently we are furthering our understanding of the inductive and effector T cell responses that develop in mice after oral immunization with *Salmonella* bearing OmpA::tgp120. We believe that these studies will provide a framework from which we will develop a prototype live oral attenuated *Salmonella*–HIV vector vaccine that can be evaluated as a candidate oral HIV T cell vaccine in volunteers.

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APPENDIX 3: RELATED PROCEEDINGS APPENDIX

None.